STUDIES ON GROWTH MEASUREMENT AND EVIDENCE FOR A POSSIBLE CARBON DIOXIDE REQUIREMENT IN TETRAHYMENA PYRIFORMIS W

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STUDIES ON GROWTH MEASUREMENT AND EVIDENCE FOR A POSSIBLE CARBON DIOXIDE REQUIREMENT IN *TETRAHYMENA PYRIFORMIS* W

By

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AN ABSTRACT

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ABSTRACT

This investigation was undertaken in order to reevaluate several of the growth measuring devices used in microbiology and to utilize the findings in an attempt to examine the problem of carbon dioxide fixation in the protozoan, *Tetrahymena*.

Cultures of *Tetrahymena pyriformis* W were grown in several types of bactone and chemically defined media. In some cases, growth of the populations was estimated by optical density (Klett-Summerson photometer), direct cell count (Sedgewick-Rafter chamber) and dry-weight determinations. Direct cell counts were also made on clone cultures grown in Van Tieghem chambers. In some cases the Van Tieghem chambers contained isosmotic solutions of either KOH, KCl or Na acetate as moisteners. The KOH was used to absorb carbon dioxide from the sealed chamber; isosmotic solutions of KCl or Na acetate were used as controls. A modified Van Tieghem chamber was constructed by soldering two small brass tubes into holes drilled into the opposite sides of the brass ring portion of the chamber. With suitable connections attached to the brass tubes, a constant flow of air can be drawn through the chamber; this permits one to control the gaseous environment of the culture.

Optical density measurements were not well correlated to either the direct cell counts or the estimated total mean protoplasmic area. No attempt was made to measure changes in the opacity of cells. Optical density measurements taken on the supernatant of the culture media showed a gradual increase in optical density. The increase in optical density, however, was not proportional to the number of cells. In fact, the supernatant of a culture grown in a bactone enriched (yeast extract) medium gave a lower optical density than a non-enriched bactone culture supernatant even though the maximum growth (cell count) in the enriched medium was three times as high as the non-enriched medium. Neither the red, green nor blue filter was affective in negating the optical density change which occurred in the supernatant. The supernatant of the chemically defined medium, compared to the bactone media, showed a slower and smaller change in its optical density. It is recommended that the supernatant of the culture medium be used as the standardization medium for optical density measurements. At least, it seems advisable to continually check the optical density of the supernatant for possible variation. Optical density changes which may occur in the supernatant not only can produce inaccurate growth measurements, but may provide helpful information concerning metabolism.

Good growth curves were obtained from clone cultures grown in the Van Tieghem chambers. This culture method gives accurate cell counts of live cells during the important early phases of the growth curve. Accurate cell counts can be made at maximum growth by photomicrography (Kodak XXX, 1/1000 seconds).

Growth of Tetrahymena was not suppressed when CO_2-free air was bubbled through the culture tubes. This confirms earlier investigations. However, a reduced growth rate was produced in Van Tieghem chambers containing 0.3 molal KOH. More consistent results were obtained when CO_2-free air was passed through Van Tieghem chambers containing single organisms in chemically defined medium.

Attempts to substitute various carboxylic acids (malic, succinic, fumaric, aspartic, alpha ketoglutaric) for this possible carbon dioxide requirement were unsuccessful. Tests were not made to ascertain whether or not these acids were able to penetrate the cell membrane.

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INTRODUCTION

The measurement of growth is the measurement of one of the fundamental attributes of microorganisms. As pointed out by Richards (1941, p. 517), the analysis of population growth requires knowledge of the environment, the individuals, and the interactions of each on the other. Furthermore, rarely is a single type of measurement adequate to give a picture sufficiently complete for analytical studies.

Populations of Protozoa are usually estimated by using a Sedgewick-Rafter chamber with a Whipple disc in the ocular lens of the microscope (cf. Hall et al., 1935). Elliott (1939) attempted to ascertain growth by centrifugation using a hematocrit tube fused to a centrifuge tube.

The population of pigmented or granular forms of microorganisms may be estimated from the optical density of the culture medium, by means of some device constructed to measure the transmission of light through the suspension. Various types of apparatuses have been devised to do this (cf. Richards and Jahn, 1933; Stier et al., 1934; and Mestri, 1935), but since 1935 some very excellent photoelectric colorimeters have been commercially available. Although these commercial photometers are primarily designed for chemical analysis they can be used as turbidimeters.

Monod (1949) has this comment concerning the current use of the photometer as a growth measuring device:

> The time-honored method of looking at a tube, shaking it, and looking again before writing down a + or 0 in the lab-book has led to many a great discovery. Its gradual replacement by determinations of "turbity at 16 hours" testifies to technical progress, primarily in the manufacturing and advertising of photoelectric instruments. This technique however is not, properly speaking, quantitative, since the quantity measured is not defined. It might be a rate, or a yield, or a combination of both.

Richards (1941, p. 519) points out that optical density depends on the number of organisms present, the distribution of organisms of various sizes, and their metabolic condition (e.g., storage products). Another variable, apparently usually ignored, is that the optical density might also depend upon changes which might occur in the culture supernatant due to the metabolic activity of the cells or simply due to an unstable type of culture medium.

One of the objectives of this investigation is to critically examine several of the methods currently used to measure growth of microorganisms. It was hoped that any conclusions drawn concerning the validity of any of the methods chosen for analysis could be employed in an attack upon the specific problem of carbon dioxide fixation in *Tetrahymena*.

Jahn (1935) and Pace and Ireland (1945) reported that *Tetrahymena* grew best in CO_2-free air when cultured in a 2 per cent bactone medium. On the other hand, Rahn (1941) has shown that the flagellated protozoans *Astasia longa* and *Polytomella caeca* showed a reduced growth rate if the carbon dioxide is constantly removed from the culture medium. Van Niel (1942) and Lynch and Calvin (1952) demonstrated a very high incorporation of radioactive carbon dioxide into the carboxyl group of succinic acid by *Tetrahymena*. In fact, Buchanan and Hastings (1946) state that Tetrahymena possesses one of the most active mechanisms for the assimilation of carbon dioxide of those systems so far studied.

Non-photosynthetic (heterotrophic) carbon dioxide fixation by bacteria was clearly demonstrated in the classical work of Wood and Werkman (1936). Evans and Slotin (1940) working with pigeon liver showed that this tissue could synthesize oxalacetate from pyruvate and carbon dioxide. These data were supported by the fact that the rate of synthesis of alpha ketoglutarate in pigeon liver depended on the concentration of carbon dioxide. Wood, Vennesland and Evans (1945) reported that radioactive carbon, from bicarbonate, administered to fasting rats was found in the third and fourth carbons of glucose. Calculations showed about one in every eight carbon atoms of glycogen was derived from the bicarbonate.

As pointed out by Buchanan (1946): "The detailed investigations with isotopic carbon of the utilization of carbon dioxide by bacteria and mammalian cells has removed one of the oldest distinctions between plant and animal life."

In addition to the protozoa mentioned above, heterotrophic carbon dioxide fixation has been found in the following organisms: (1) in bacteria by Slade (1942), Gitterman and Knight (1952), Tomlinson and Baker (1954), Abelson et al. (1952), Stoppani et al. (1955), Bolton et al. (1952); (2) in molds by Foster et al. (1941), Rockwell and Highberger (1927), Heplar and Tatum (1954); (3) in frogs by Cohen (1954), and Flickinger (1954); (4) in fowl by Donaldson and Marshall (1956); (5) in rats by Delluva and Wilson (1946); and (6) in rabbits by Donaldson et al. (1954).

Wood (1951) remarks as follows:

> Much remains to be done before the full significance of CO_2 fixation can be assessed in metabolism. One of the questions that remains unanswered is whether or not CO_2 fixation is an essential step in normal heterotrophic metabolism or whether the occurence of fixed CO_2 is due largely to side reactions which are not required for metabolism.

Krebs (1951) has estimated that, for mammalian cells, the amount of carbon dioxide which reeneters metabolism by fixation is less than 10 per cent of that formed. As pointed out by Kidder (1951, p. 395) Tetrahymena (and probably ciliates in general) possess enzyme systems somewhat comparable to those of higher animals. One would suspect, therefore, that if a carbon dioxide requirement does exist for Tetrahymena it would not be of the order of magnitude as that found in bacteria and molds.

The objectives of this investigation are twofold: (1) to reexamine several of the current methods used for growth measurement in order to evaluate them according to their ability to measure various aspects of growth, and (2) to utilize these growth measuring devices in an attempt to ascertain whether or not carbon dioxide is required for growth in Tetrahymena pyriformis W.

MATERIALS AND METHODS

A. NON-CHEMICALLY DEFINED MEDIA (BACTONE MEDIA E)

One of the two general culture media that was used in this study is a non-chemically defined medium whose basic constituent is Bacto-Tryptone (Difco Laboratories, Detroit, Michigan). Bacto-Tryptone, as stated by the Difco Laboratories, contains proteoses, peptones, cystine, tyrosine, tryptophane, iron, magnesium, calcium, potassium, sodium, chlorine, phosphorous, and sulfur. Fourteen per cent of the powder is nitrogen. A one per cent solution of Bacto-Tryptone after fifteen minutes autoclaving has a pH of 7.2.

Although a one per cent Bacto-Tryptone solution with no additional metabolites will support a growth of about 180 cells per cubic millimeter for about two weeks, the addition of a buffer and thiamine greatly increases the total number of cells (350 cells per cubic millimeter) as well as the duration of the culture.

The bactone medium (hereafter designated as bactone medium E) is essentially that used by Dr. James F. Hogg (personal communication) and consists of the following: Bacto-Tryptone 10.0 grams; yeast extract 0.1 grams; glucose 1.0 grams; K_2HPO_4 1.0 grams; KH_2PO_4 1.0 grams; thiamine HCl 0.002 mg.; sodium acetate 1.0 grams; and H_2O 1000 ml. This medium is adjusted to pH 7.2 using 0.1N NaOH and autoclaved at 15 pounds pressure for 15 minutes.

B. CHEMICALLY DEFINED MEDIA

The chemically defined media used in this study are those of Elliott et al. (1954) and Kidder and Dewey (1951, p. 392). The constituents of these media are given in Table I. The vitamins are kept in a stock solution of 25 per cent alcohol in such concentration that one milliliter is used per liter of medium. The stock solution of salts, excluding KH_2PO_4 and K_2HPO_4, are concentrated so that 50 milliliters of the stock solution will fulfill the salt requirement for one liter of medium. The KH_2PO_4 and K_2HPO_4 are kept in separate stock solutions and added separately because these two salts will form an insoluble precipitate if mixed with the other salt stock solution. The dextrose is autoclaved separately as recommended by Kidder (1951). After the pH is adjusted to 7.4 using 0.1N NaOH, the chemically defined medium is autoclaved for 15 minutes at 15 pounds pressure.

The bactone medium as well as the synthetic media are autoclaved in the vessels which are to be used in the experiment. Stock cultures are grown in test tubes (20 x 2.5 cm.) held in a vertical position. All vessels containing autoclaved medium which is not to be used immediately, are sealed with Parafilm and stored in the refrigerator.

1. Indirect cell counts.

Photoelectric colorimetry. Growth curves were established using a Klett-Summerson Photoelectric Colorimeter. A blue filter (Klett #42;400-500 millimicrons) was used as recommended by Elliott (1949) and, in some cases, the red filter (Klett #66;640-700 millimicrons) was used as recommended by Kidder (1951, p. 395); additional measurements

TABLE I

CHEMICALLY DEFINED MEDIA

Compounds	Elliott Medium (micrograms per milliliter)	Kidder and Dewey Medium 2c (micrograms per milliliter)
DL Alanine	none	1100
L Arginine	150	860
L Aspartic Acid	none	1220
Glycine	none	100
L Glutamic Acid	none	2330
L Histidine	110	420
DL Isoleucine	100	1260
L Leucine	70	1940
L Lysine	35	1520
DL Methionine	35	680
L Phenylalanine	100	1000
L Proline	none	1754
DL Serine	180	1540
DL Threonine	180	1760
L Tryptophane	20	240
DL Valine	60	1320
Ca pantothenate	0.10	0.50
Nicotinamide	0.10	0.50
Pyridoxine HCl	2.00	5.00
Pyridoxal HCl	none	0.50
Pyridoxamine HCl	none	0.50
Riboflavin	0.1	0.50
Pteroylglutamic acid	0.01	0.05
Thiamine HCl	1.00	5.00
Biotin	none	0.0025
Choline chloride	none	5.00
Thioctic acid[a]	0.001	0.001[b]
Adenylic acid	25.0	200.0
Cytidylic acid	25.0	250.0
Guanylic acid	25.0	300.0
Uracil	25.0	100.0
$FeSO_4 \cdot 7H_2O$	0.5	none
$MgSO_4 \cdot 7H_2O$	10.0	140.0
$Fe(NH_4)_2(SO_4)_3 \cdot 6H_2O$	none	62.5
$MnCl_2 \cdot 4H_2O$	none	1.25

TABLE I - Continued

Compounds	Elliott Medium (micrograms per milliliter)	Kidder and Dewey Medium 2c (micrograms per milliliter)
$ZnCl_2$	none	0.125
$CaCl_2 \cdot 2H_2O$	none	30.0
$CuCl_2 \cdot 2H_2O$	0.5	3.0
$FeCl_3 \cdot 6H_2O$	none	0.75
K_2HPO_4	100.0	500.0
KH_2PO_4	none	500.0
$Zn(NO_2)_2 \cdot 6H_2O$	5.0	none
Dextrose	1000	2500
Sodium acetate	1000	none

[a] Protogen was used in the original medium.

[b] Na DL Thioctate was kindly provided by Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, New York.

were made with a green filter (Klett #54;500-570 millimicrons). Klett calibrated test tubes were used and were checked for accuracy. In some cases the cultures were grown directly in the Klett tubes, but in most cases they were grown in 125 milliliter pyrex Erlenmyer flasks and then transferred to the Klett tubes for measurement. After the instrument is adjusted using distilled water, the optical density of the culture is compared to the "blank standard." The "blank standard" in these experiments was either: (a) fresh culture medium in which no organisms had ever been grown or (b) the supernatant of the medium in which the organisms had been grown. When the "blank standard" was as is stated in (b) above the organisms were first centrifuged at moderate speed for 15 minutes and the supernatant immediately drawn off using a 10 milliliter syringe. To facilitate the removal of the supernatant a 2 inch length of plastic tubing was fastened to the end of the syringe to be used in place of a needle. The removal of the supernatant must be carried out immediately upon completion of centrifugation because the organisms will quickly migrate back into the supernatant. In fact, the supernatant was drawn off as soon as the tubes came to a stand still, but before the tubes were removed from the centrifuge. This procedure produced a cell-free supernatant.

In some cases the supernatant, after centrifugation, was filtered using a Seitz vacuum type filter with a low vacuum. Because of their very elastic cell membrane, Tetrahymena can not be filtered out using ordinary filter paper. All glassware used in this study was cleaned with potassium dichromate cleaning solution and well rinsed with large quantities of tap water followed by several final rinsings of distilled water. As reported by Richards (1936), unless all glassware cleaned with acid solutions is thoroughly rinsed, serious errors may enter into the experiments in which the glassware is used. For this reason a rack was constructed which held the Erlenmyer flasks in an inverted position over a stream of water directed upward and thus onto the bottom of the flask. In this way, a continuous supply of fresh water is flushed through the inside of the flask.

Dry-weight determinations. A measured volume of the culture was centrifuged and the supernatant drawn off with a syringe as described in the previous section. The cells were washed with distilled water to remove any remaining medium and recentrifuged. This process was repeated three times to insure the complete removal of medium. The cells were then transferred to a weighing bottle and dried for twenty-four hours in a drying oven at 100°C. The weighing bottles were allowed to cool in a desiccator before final weighings were made. Heating was continued until a constant dry-weight was obtained. The amount of original culture used, of course, depended upon the age of the culture. A three day old culture, for example, required a much larger volume of culture in order to obtain a weighable dry-weight of organisms. Results are always reported as milligrams dry-weight per milliliter of culture.

2. Direct cell count.

The Sedgewick-Rafter counting chamber. Direct cell counts can be made using the Sedgewick-Rafter counting chamber along with the Whipple Micrometer. The technique is similar to that used by Hall *et al.* (1935) and by Whipple (1927).

One milliliter is withdrawn from the growing culture. One to several milliliters, depending upon the age of the culture, of 10 per cent formalin is added to the original one milliliter. The formalin solution thus dilutes the culture as well as kills the cells. One milliliter of the diluted, dead cells is then transferred to the Sedgewick-Rafter counting chamber and the cells allowed to settle to to the bottom. Using a 10x objective lens and a 10x eyepiece, the largest dimensional area circumscribed by a Whipple Micrometer is one square millimeter. Since the Sedgewick-Rafter counting chamber has a depth of one millimeter, the cells present on one square millimeter of the bottom actually represent the cells that have settled out of one cubic millimeter. The Dilution Factor is equal to the sum of the original one milliliter of cells plus the number of milliliters of formalin which were added as a "fixing-dilution" agent. Therefore, the total number of cells per cubic millimeter is equal to the actual count of the cells per cubic millimeter multiplied by the Dilution Factor. The counting chamber holds 1,000 cubic millimeters and 10 individual areas occupying one cubic millimeter each are chosen at random and an average of these 10 areas is used as the cell count prior to multiplication by the Dilution Factor.

The Van Tieghem chamber. The Van Tieghem chamber as described by Duggar (1909) is a type of moist-chamber preparation most often used for cultures of fungi. These chambers are also very adaptable to the growth of protozoa and can be used very nicely to obtain direct cell counts for establishing accurate growth curves. Since a thorough account of the procedure for making the Van Tieghem chamber could not be found in the literature, the method had to be worked out by the author. Some of the important steps are given in detail.

The main constituents of the Van Tieghem chamber are a microscope slide, a cover glass and a metal or glass ring about 10 millimeters high and 10 millimeters inside diameter. The ring and microscope slide can be sterilized in a Petri dish using dry heat. The ends of the glass ring are then touched to the surface of melted vaseline. This technique produces a neat, even layer of vaseline on both ends of the ring. The ring can be placed on the center of the slide while the vaseline is still liquid thus a good seal is obtained between the ring and the slide. The ring, sealed to the slide, is then returned to the Petri dish and stored ready for use.

The cover glasses are very important since they are the objects which actually hold the culture and thus must be perfectly clean and sterile. The cover glasses are allowed to soak in absolute alcohol for several weeks after being cleaned in acid cleaning solution and thoroughly rinsed. They are then individually rinsed, using a small forceps, in separate beakers of absolute alcohol or metasilicate as suggested by White (1954) and allowed to air dry. A very satisfactory rack for drying large quantities of cover glasses can be constructed by tacking a small coil spring (10 centimeters long and 0.75 centimeters in diameter) which has been stretched beyond its elastic limit (to about 20 centimeters in length) to a 10 inch board. A piece of absorbent paper may be placed between the spring and the board; the cover glasses are then placed between the loops of the extended spring. In this way the cover glasses dry quite free of lint and water marks because they are adequately separated from each other. After they are dry, all of the cover glasses are placed in one clean Petri dish or a weighing bottle and dry-heat sterilized. After sterilization it is important that the cover glasses be cooled very slowly. In fact, allowing them to remain in the oven as the oven cools is very satisfactory. If the cover glasses are cooled rapidly, much dust enters the Petri dish, as the volume of air contracts, and deposits on the cover glasses. This dust not only destroys the sterility but inhibits the proper drop formation when the culture is made. If the cover glass contains anything on its surface which gives it a wettable surface it is impossible to form a satisfactory hanging drop culture for the drop will tend to spread and to coalesce with the small droplets deposited from the moistening material in the bottom of the chamber. After it has cooled to room temperature, the Petri dish with the enclosed cover glasses should be kept in a vaseline-sealed desiccator to insure cleanliness and prolong sterility. The cover glasses are resterilized every few days. It is not satisfactory to flame the cover glass just prior to the placing of the culture drop because a wettable surface, presumably from deposited carbon, results.

Most of the Van Tieghem chambers used in this study contained hanging drops which possessed only one organism at the beginning of the experiment. The following procedure was found to be the most satisfactory for isolating single organisms and at the same time maintaining the best conditions for sterility. Five milliliter aliquots of media are autoclaved in 10 milliliter Erlenmyer flasks. Just prior to setting up an experiment, the 5 milliliter aliquot is inoculated using

a flame-sterilized transfer needle in which the wire had been bent to form a loop having a diameter of about 2 millimeters.

The following equipment and materials have been found necessary for the setting up of Van Tieghem cultures in quantity:

a microscope equipped with a 48 millimeter objective lens and a 10x ocular lens or a 32 millimeter objective lens used with a 6x ocular lens;

a sufficient quantity of sterilized units, as mentioned previously, consisting of a Petri dish containing a slide with the glass ring sealed to it by vaseline. The free end of the ring has also been previously coated with vaseline;

a supply of clean, sterile cover glasses;

a transfer needle containing a loop having a diameter of about 2 millimeters; and

a glass ring which has been permanently cemented to a microscope slide.

The procedure for setting up the Van Tieghem culture is as follows. The unit consisting of a glass ring cemented to a slide is passed through a flame and allowed to cool. A sterile cover glass is then placed on top of the ring and a drop of culture medium from the previously inoculated 5 milliliter aliquot is placed on the cover glass using the small sterile transfer loop. The cover glass is quickly inverted so that the drop extends down into the hollow of the glass ring. Here again, if the cover glasses are not perfectly clean the drop will spred as the cover glass is inverted. The entire drop can now be examined under the microscope if the proper objective and ocular lens combination is used. In this way one can select the drop or reject it before sealing the cover glass to a permanent Van Tieghem chamber. The drop, upon this trial inspection, may contain none to several organisms depending upon the size of the original inoculum. In general, it was found that if the 5 milliliter aliquot is inoculated by the transfer of a single 2 millimeter loop from a stock culture of maximum growth, the drops withdrawn from the aliquot would stand the best chance of containing only one organism. This procedure, of course, is best adjusted to meet the needs of the experiment. Usually 5 or 6 drops had to be examined for every one that was selected. This is somewhat time consuming but is necessary if all of the drops are to contain an equal number of organisms. Once the drop is selected, the cover glass is then transferred to the Van Tieghem chamber which had been previously prepared. If desirable, a few drops of a suitable moistener may be added to the bottom of the chamber to insure adequate moisture. Cultures of Tetrahymena can be grown without any additional moistener, but for some purposes the moistener may be desirable. The choice of the moistener is of utmost importance because of the thermal distillation of water which will occur between the moistener and the culture drop. A few drops of the same medium of which the culture drop consists is the most osmotically suitable. However, it is liable to become contaminated so that the choice of a suitable moistener is best made by examining various salt solutions which contain no nutrients. If it is possible to calculate the ionic strength of the culture media, a solution of equal osmotic activity of some non-volatile salt will serve as a good moistener. For non-chemically defined media, such as a bactone medium, the proper concentration for the moistener can best be found empirically. This is done most easily be setting up a series of hanging drops of pure medium, containing no organisms, over moisteners of varying concentrations. The diameter of the drop can be measured using an ocular micrometer. The drop will increase in size if the ionic strength of the moistener is less than that of the culture medium. The drop will decrease in size if the ionic strength of the moistener is greater than that of culture medium. A moistener which has an ionic strength which is greater than that of the medium is to be avoided for the withdrawal of water from the culture medium due to distillation has a strong inhibitory effect on the growth of the culture. Moisteners which have an ionic strength less than that of the medium lose water to the culture medium thereby diluting it. However, this effect is not inhibitory for excellent growth can be obtained even if distilled water is used as a moistener.

The problem of carbon dioxide fixation as approached in some parts of this particular study, involved the use of ionized moisteners. It was necessary that the moisteners chosen, although they might have certain chemical properties which were greatly different, must possess identical osmotic properties. Thus in the choice of moisteners one must carefully consider their Practical Osmotic Coefficients. The Practical Osmotic Coefficient (ϕ) can be obtained by isopiestic measurements which are made in an apparatus, which in principle, is very similar to the Van Tieghem chamber. Robinson and Stokes (1955) describe an isopiestic measurement as follows:

> Let X and Y be two solutions initially at the same temperature, the vapor pressure of X being initially greater than that of Y and let them be connected by a path through which vapor can pass. The solvent will distil from solution X to solution Y, resulting in a cooling of X and a heating of Y from the heat of vaporization generated during the process. Because of these temperature changes the vapor pressure of X decreases and that

of Y increases and if perfect thermal insulation could be maintained between the two solutions, a steady state would be set up with a temperature difference between the two solutions sufficient to equalize the vapor pressures.

If thermal conductance is allowed however, for example starting with two solutions each containing one gram of water and sufficient Na and KCl to make each solution 0.4 molal, the distillation of 61 mg. of water will concentrate the KCl solution to 4.260 molal and dilute the NaCl to 3.770 molal at which concentrations the vapor pressures are equal.

The Practical Osmotic Coefficients taken from Conway (1952) for KOH, KCl and $NaC_2H_3O_2$ are given in Table II.

The following formula (see Harned and Owen, 1950) expresses the relationship of the molality (m) of the salt to the activity of the water (a_w):

$$RT \ln a_w = \frac{-RT}{55.5} (\phi m)$$

where R is the gas constant, T the absolute temperature and ϕ the Practical Osmotic Coefficient.

Since it is the activity of the water which must be taken into consideration in the Van Tieghem chamber, the following relationship can be shown for the comparison of two salts:

$$\text{for salt \#1, } \log_{10} a_w = \frac{1}{2.303 \times 55.5} (\phi m)$$

$$\text{for salt \#2, } \log_{10} a'_w = \frac{1}{2.303 \times 55.5} (\phi' m')$$

If a_w must equal a'_w, which is the requirement for a proper osmotic control, then:

$$\frac{1}{2.303 \times 55.5} (\phi m) = \frac{1}{2.303 \times 55.5} (\phi' m')$$

and $$\phi m = \phi' m'$$

TABLE II

PRACTICAL OSMOTIC COEFFICIENTS (ϕ)
(AFTER CONWAY, 1952)

Molality	$NaC_2H_3O_2$	KOH	KCl
0.1	0.940	0.944	0.927
0.2	0.939	0.936	0.913
0.3	0.945	0.938	0.906
0.4	0.951	0.944	0.902
0.5	0.959	0.953	0.899
0.6	0.967	0.962	0.898
0.7	0.977	0.972	0.897
0.8	0.986	0.983	0.897
0.9	0.994	0.993	0.897
1.0	1.002	1.003	0.897

Therefore, in order to find two solutions which have an equal osmotic effect upon the distillation of water, one must find the molalities at which the value of ϕm is the same. Table III gives the values of ϕ, m and ϕm for KOH, KCl and $NaC_2H_3O_2$ used in these experiments.

The best way to find the proper molalities at which two salts have equal water activity is to plot a graph of the molality versus the product of the molality and the practical osmotic coefficient. This relationship is given in Figure 1. One can see from this graph, for example, that a 0.3 molal solution of KOH would have the same osmotic effect as a 0.31 molal solution of KCl.

D. STUDIES ON CARBON DIOXIDE REQUIREMENT

1. Aeration flask experiments.

The apparatus as pictured in Plate 1 was used in several experiments in an attempt to remove carbon dioxide from the culture medium. Atmospheric air was bubbled, using a vacuum system, first through a 20 per cent KOH solution and then through a tube of 8-20 mesh Ascarite before entering the medium itself. Fritted gas dispersion cylinders were used in the culture medium as well as in the KOH solutions in order to decrease the bubble size and thereby increase the absorption area. The inlet and outlet for air entering the 20 x 2.5 centimeter test tubes used as culture tubes contained sterile cotton. The culture tubes with cotton filled drying tubes attached were autoclaved as a unit to insure sterile conditions. Air for the control tubes was bubbled through water instead of KOH.

TABLE III

VALUES OF THE PRODUCT OF THE PRACTICAL OSMOTIC COEFFICIENT (ϕ) AND MOLALITIES FOR KCl, KOH AND $NaC_2H_3O_2$

Molality (m)	$NaC_2H_3O_2$		KOH		KCl	
	ϕ	ϕm	ϕ	ϕm	ϕ	ϕm
0.1	0.940	0.0940	0.944	0.0944	0.927	0.0927
0.2	0.939	0.1878	0.936	0.1872	0.913	0.1826
0.3	0.945	0.2835	0.938	0.2814	0.906	0.2718
0.4	0.951	0.3804	0.944	0.3776	0.902	0.3608
0.5	0.959	0.4795	0.953	0.4765	0.899	0.4495
0.6	0.967	0.5902	0.962	0.5772	0.898	0.5388

FIGURE 1

The relation between the molality and the product of the molality and the osmotic coefficient for KCl, KOH and $NaC_2H_3O_2$.

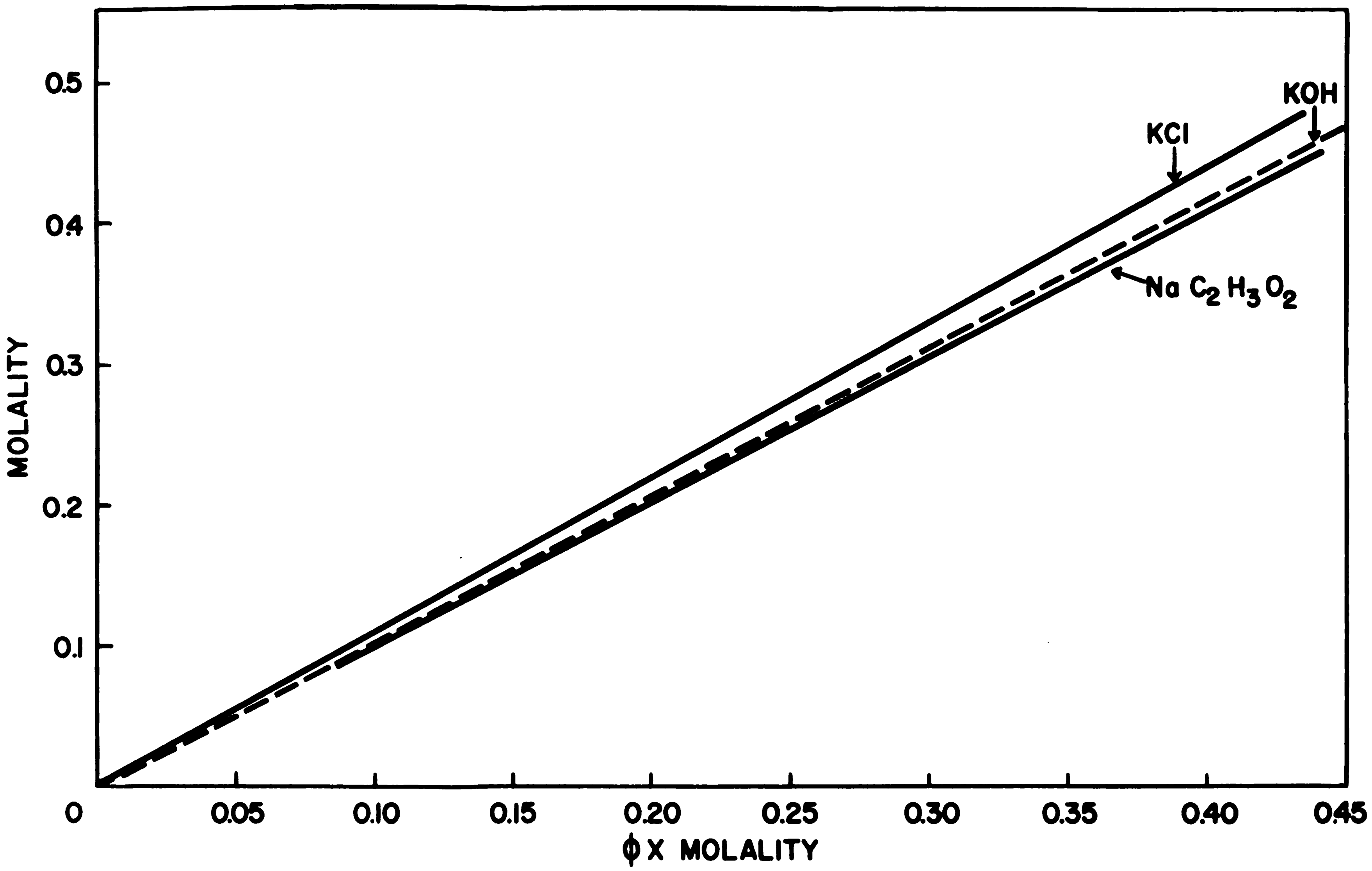
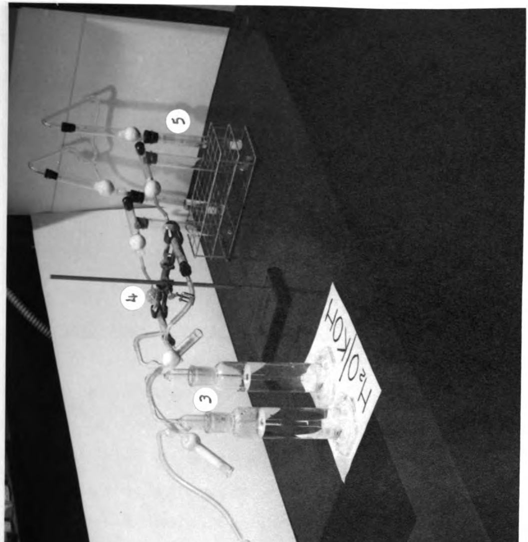

MOLALITY
0.5
0.4
0.3
0.2
0.1
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
ϕ X MOLALITY
KCl
KOH
Na $C_2H_3O_2$

PLATE 1

The apparatus used in the aeration-flask experiments.

Legend:

1. Tubing leading to the vacuum pump;
2. culture tubes (control);
3. gas washing bottles;
4. Ascarite tube; and
5. culture tubes (experimental).


1
2
3
4
5
H_2O/KOH

1. Te

ector

Sever

scale

[illegible]

four

organ

2. F

[illegible]

[illegible]

The

[illegible]

[illegible]

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[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

2. Desiccator-depression slide experiments.

Another method used to remove carbon dioxide involved a desiccator (16 centimeter diameter) in which a depression slide was placed. Several small vessels containing 20 per cent KOH were placed in a sealed desiccator and a depression slide containing a drop of inoculated medium was placed between the vessels containing the KOH. At the end of four days the depression slide was removed from the desiccator and the organisms were counted under the microscope.

3. Petri dish-depression slide experiments.

Six or seven microscope slides are stacked horizontally in a Petri dish, the top slide being a depression slide. A 20 per cent solution of KOH is pipetted into the space around the slides and then the Petri dish cover is sealed with vaseline on the inside, or a layer of Parafilm is wrapped around the outside. The culture drop located within the depression of the uppermost slide can be viewed through the Petri dish cover with a 48 millimeter objective lens and a count of the organisms taken.

4. Van Tieghem chamber experiments.

The Van Tieghem chambers as described in the previous section were used in the carbon dioxide experiments. An attempt was made to remove all carbon dioxide by placing the proper molalities of KOH in the bottom of the chamber. Slight modifications were made by increasing the volume of KOH until it nearly filled the chamber in which case the hanging drop under the cover glass occupied the air space formed by the meniscus of the KOH solution. In other cases, when only a few drops of moistener were used, 8 x 12 millimeter pieces of filter paper were inserted in a vertical position within the chamber to facilitate the absorption of the carbon dioxide by the KOH. Several experiments were conducted in which the Van Tieghem chambers with added filter paper were placed in a Dubnoff Metabolic Shaking Incubator. The incubator portion of the apparatus was not utilized because room temperature was sufficient. The cultures, however, were kept covered and the apparatus set at a rate producing one complete oscillation per second.

A basic alteration of the Van Tieghem chamber is pictured in Plate 4. A brass ring with a 10 millimeter inside diameter and a height of 10 millimeters is used for the main chamber. Two small holes were drilled on opposing sides of the ring and a one centimeter piece of brass tubing having a diameter of 2 millimeters was soldered into each hole. This chamber, sealed to a microscope slide with vaseline, can be clamped to a microscope using two Double Clamps #204 (Harvard Apparatus Company). Using a small vacuum and the necessary hose connections the air can be made to pass through the chamber after the cover slip is sealed in place with vaseline. To inhibit desiccation of the drop due to the continual passage of air, the air is drawn through distilled water before it enters the chamber and a few drops of moistener are placed in the bottom of the chamber at the beginning of the experiment. To remove the carbon dioxide from the incoming air, the air is bubbled through two Erlenmyer flasks containing 100 cubic centimeters of 20 per cent KOH and then through a 6 inch tube of 8-20 mesh Ascarite. A tube containing sterile cotton is placed at the incoming end of the gas chain. The air, after passing through the Ascarite, is bubbled through distilled water before it enters the chamber. This apparatus is pictured in Plates 2 and 3. The passage of air can be controlled by a hose clamp placed between the chamber and the vacuum pump. This procedure enables one to carefully control the rate of air flow so that the experiments can be well controlled. The rate of air flow for these experiments was usually held at about 160 bubbles per minute. This rate is equivalent to about 20 cubic centimeters per minute. If the volume of the chamber, with a few drops of water in the bottom, is estimated at about 0.5 cubic centimeters, this rate of air flow would change the air in the chamber once every 1.5 seconds.

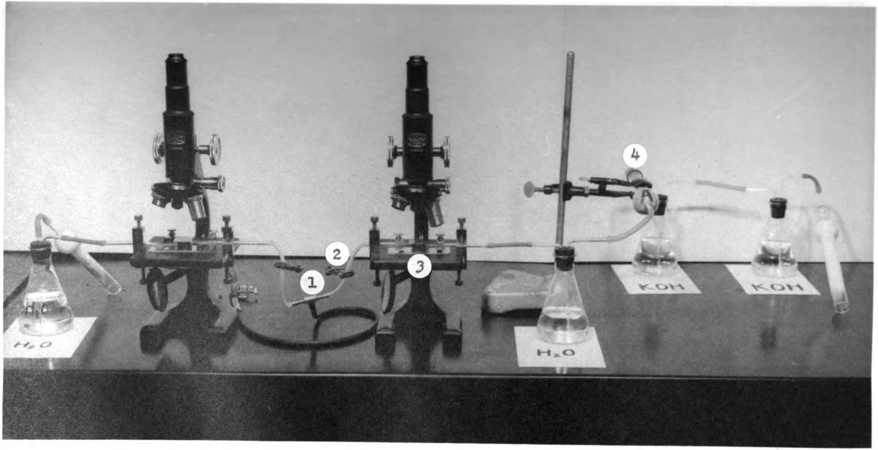
Plate 4 shows the usual type of Van Tieghem chamber on the left and the modified chamber containing the adaptation for continual air passage on the right.

PLATE 2

Modified Van Tieghem chamber apparatus.

Legend:

1. Tubing which leads to vacuum pump;
2. clamp used to regulate air flow;
3. Van Tieghem chamber; and
4. Ascarite tube.



H_2O
1
2
3
H_2O
4
KOH
KOH

PLATE 3

Modified Van Tieghem chamber apparatus (detail).

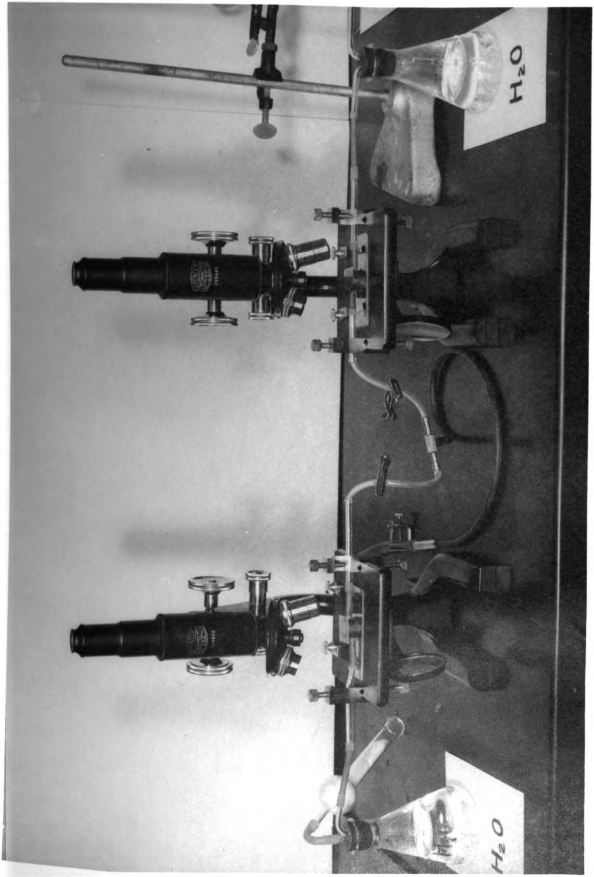

H₂O
H₂O

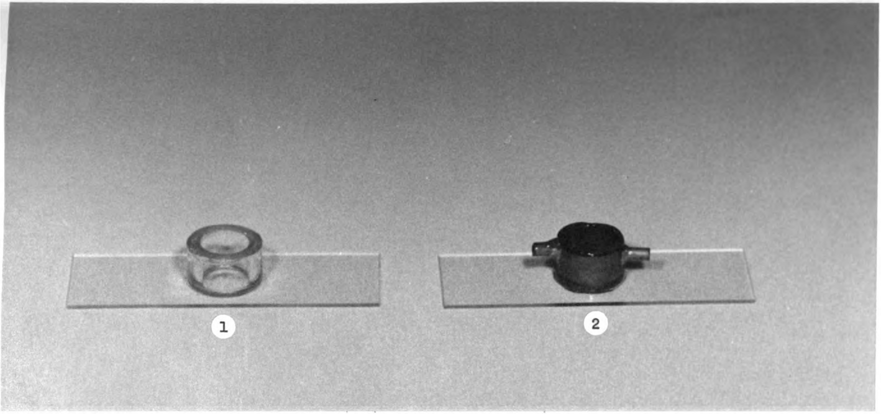
PLATE 4

The Van Tieghem chambers.

Legend:

1. Ordinary Van Tieghem chamber consisting of a glass ring sealed to a microscope slide with vaseline; and

2. modified Van Tieghem chamber containing small tubes soldered to a brass ring.


1
2

RESULTS

A. OBSERVATIONS ON GROWTH IN *TETRAHYMENA*

1. The relationship between the direct cell count, optical density and the total mean protoplasmic area.

Organisms used in this experiment were cultured in a one per cent bactone medium, and in thiamine enriched (0.2 gamma per milliliter) bactone medium. Estimates of growth were made with a Sedgewick-Rafter counting chamber, and a Klett-Summerson photoelectric colorimeter fitted with a blue filter. An ocular micrometer was used for ascertaining length and width measurements. For details concerning procedures for making measurements and recording data, consult Tables IV and V.

Table VI suggests that the error in the various measurements (mean length, mean width, etc.) varied from about 1.5 to 6.4 per cent when the number of cells measured was decreased from 270 to 20.

Table VII summarizes the data obtained in this series of experiments.

It is evident from Table VII that a maximum of 192 cells per cubic millimeter was obtained in the bactone medium (144 hour culture) which was equivalent to a Klett scale reading of 24.5. On the other hand, the maximum cell count using the bactone enriched medium (168 hours) increased to 273 cells per cubic millimeter which was equivalent to a Klett scale reading of 55.5. Thus, the ratio between maximum *numbers* of cells in the bactone enriched medium (273 cells per cubic

TABLE IV

AN EXAMPLE OF THE DATA SHEET USED IN PART 1

Experiment. . . #1
Culture Media . 1% bactone
Date. 8/15
Hour. . . . . . 10 a.m.
Total Hours . . 73

A. Direct Cell Count

Trial Number[a]	Sample #1	Sample #2
1	62	85
2	66	74
3	70	67
4	68	88
5	79	75
6	64	105
7	52	67
8	69	85
9	76	89
10	74	78
Mean	68	81
General mean	74	
Dilution factor[b]	2	
Cells/mm^3	148	

B. Photometric Measurements

	Sample #1	Sample #2
Scale Reading	21	24
Mean scale reading	22.5	
Optical density[c]	0.045	

C. Cell Dimensions

Cell	Length[d]	Width[d]
1	45	21
2	34	18
3	44	20
4	46	21
5	43	17
6	50	23
7	44	20
8	47	16
9	53	19
10	51	22
11	55	23
12	42	17
13	46	20
14	44	21
15	42	21
16	45	20
17	56	25
18	56	23
19	42	21
20	53	20
Mean	46.9	20.4
Calibration factor[e]	0.94	0.94
Mean (microns)	44.0	19.1
Area[f] per cell	2,604	
Area[g] per mm^3	385,392	

---

[a] Trial number: Ten microscopic fields were chosen from each of the two samples.

[b] Dilution factor: This term was described under the discussion of the Sedgewick-Rafter chamber in the previous section. Specifically, it is the number which when multiplied by the number of cells counted will give the corrected total of cells per cubic millimeter.

TABLE IV - Continued

[c] Optical density: The optical density equals 0.002 multiplied by the scale reading.

[d] Length and width measurements: These values are expressed in units of the ocular micrometer. The calibration factor is discussed in footnote "e" below.

[e] Calibration factor: One micrometer unit equals 0.00094 millimeters or 0.94 microns.

[f] Area of cell: On the assumption that the area of the cell can best be calculated as the area of an ellipse, the "protoplasmic area" of the cell was calculated as follows: Area of the cell $= \pi ab$, where "a" and "b" are the semiaxes of the elipse, or in this case, the length and width of the cell.

[g] Area per mm^3: Mean area per cell multiplied by the total cells per cubic millimeter will give the total protoplasmic area in square microns per cubic millimeter.

TABLE V

AN EXAMPLE OF THE DATA SHEET USED IN PART 1[a]

Experiment. . . #1
Culture Media . 1% bactone plus thiamine (0.2 gamma per milliliter)
Date. 8/20
Hour. . . . . . 10 a.m.
Total Hours . . 193

A. Direct Cell Count

Trial Number	Sample #1	Sample #2
1	97	94
2	89	81
3	96	66
4	91	60
5	107	102
6	78	81
7	84	89
8	113	77
9	85	130
10	74	101
Mean	91.4	88.1
General mean	89.7	
Dilution factor	3	
Cells/mm^3	269	

B. Photometric Measurements

	Sample #1	Sample #2
Scale reading	50	53
Mean scale reading	51.5	
Optical density	0.103	

C. Cell Dimensions

Cell	Length	Width
1	52	24
2	42	18
3	46	22
4	46	24
5	51	25
6	36	17
7	56	25
8	49	24
9	48	24
10	43	20
11	43	16
12	38	19
13	42	20
14	54	24
15	47	22
16	51	26
17	45	21
18	47	23
19	40	20
20	43	23
Mean	45.9	21.8
Calibration factor	0.94	0.94
Mean (microns)	43.1	20.5
Area per cell	2,739	
Area per mm^3	736,791	

---

[a] Explanations for the terms used in this table can be found in the footnotes to Table IV.

TABLE VI

A COMPARISON OF DATA TAKEN ON 20 CELLS WITH THE SAME DATA TAKEN ON 270 CELLS

	20 Cells	270 Cells	Per cent Difference
Mean length	47.8	44.9	6.4
Mean width	22.2	21.2	4.7
Mean area	1067.7	963.5	4.5
Mean $\frac{\text{Length}}{\text{Width}}$	2.16	2.13	1.5

TABLE VII

A SUMMARY OF THE DATA COLLECTED IN PART 1

Bactone Media[a]							Bactone Media Enriched With Thiamine[b]						
Age of Culture in Hrs.	Cells per mm^3	Scale Reading	Optical Density	Cell Length	Cell Width	Area[c] per mm^3	Age of Culture in Hrs.	Cells per mm^3	Scale Reading	Optical Density	Cell Length	Cell Width	Area[c] per mm^3
73	148	22.5	0.044	44.0	19.1	0.385	73	216	35.5	0.071	46.3	20.3	0.629
96	157	27.5	0.054	43.8	19.4	0.413	96	253	44.0	0.088	46.1	20.9	0.756
144	192	24.5	0.048	36.1	16.4	0.352	144	270	54.0	0.108	49.6	22.3	0.925
168	161	21.0	0.042	35.0	16.3	0.286	168	273	55.5	0.111	44.9	20.8	0.790
192	101	18.5	0.036	30.9	16.3	0.159	192	269	51.5	0.103	43.1	20.5	0.736
216	95	16.5	0.032	30.4	15.1	0.134	216	275	50.5	0.101	43.8	18.9	0.707
240	70	17.0	0.034	31.8	15.7	0.110	240	265	54.5	0.109	45.1	19.5	0.722
264	44	17.0	0.034	23.3	13.0	0.042	264	258	54.0	0.108	38.0	19.6	0.595
312	46	17.0	0.034	15.8	15.6	0.036	312	149	40.0	0.080	41.8	21.1	0.407
336	28	20.0	0.042			0.035							
							360	130	38.0	0.076	39.7	18.7	0.299
							408	72	37.0	0.074	40.5	21.9	0.196

[a] A 1% Bacto-tryptone solution (i.e., 1 gram Bacto-tryptone plus 99 grams of water).

[b] A 1% bactone solution enriched with thiamine (0.2 gamma per milliliter).

[c] See footnote "g" to Table IV.

millimeter), and the non-enriched bactone medium (192 cells per cubic millimeter) was 1.4. On the other hand, the ratio between Klett scale readings (55.5 to 24.5) in these two media was 2.3. If a direct proportion had existed between these two measurements, i.e., cell counts and Klett scale readings, the two ratios described above would have been equal. It is also evident from columns two and three of Table VII, that for cell counts of 70, 44 and 46 cells per cubic millimeter, a Klett scale reading of 17 was obtained in all three cases.

In the bactone enriched culture medium, a minimum of 72 cells per cubic millimeter and a Klett scale reading of 37 were found at 408 hours subsequent to inoculation. This Klett scale reading was essentially the same as the scale reading obtained at 73 hours at which time there were 216 cells per cubic millimeter. These observations suggest that optical density measurements (Klett scale readings) of culture solutions and the number of organisms per cubic millimeter of culture solutions are not closely correlated.

When the enriched bactone medium was used, the Klett scale reading at 73 hours was 35.5 and the total protoplasmic area of organisms was calculated to be 0.629 square microns per cubic millimeter. At 408 hours when the Klett scale reading was nearly the same, the total protoplasmic area was only 0.196 square microns per cubic millimeter. A similar situation exists when the non-enriched bactone is used. For example, although the scale readings obtained at 216, 240 and 264 hours were identical, the total mean protoplasmic area has decreased from 0.110 to 0.036. The observations just cited clearly indicate that the Klett scale reading and the total protoplasmic area are not correlated.

As one analyzes the data in Table VIII, it is obvious that the Klett scale reading when used to measure the progress of growth in time, is not directly proportional to either the direct cell count or the protoplasmic area. However, the ratio of the Klett scale reading to the total mean protoplasmic area is nearly constant in the enriched bactone medium for the first 216 hours of growth. This was not the case for the non-enriched bactone medium because this same ratio increases from 57.14 to 119.40 during the first 216 hours.

One might suspect from the preceding observations, since neither the cell count nor the total mean protoplasmic area were directly proportional to the Klett scale reading, that perhaps there are additional factors within the culture medium which can affect the optical density of the culture medium.

2. Klett scale readings of various portions of bactone and bactone enriched media.

The media used in this experiment are the same as in Part 1, namely, a one per cent bactone medium, and a one per cent bactone medium enriched with thiamine (0.2 gamma per milliliter). The following data were collected in this experiment: (a) the Klett scale reading of the culture medium containing cells using fresh culture medium as the standard, $C^{(M)}$; (b) the scale reading of the culture medium containing cells using fresh culture medium that had been filtered as the standard, $C^{(FM)}$; (c) the scale reading of the fresh culture medium using the filtered fresh culture medium as the standard, $M^{(FM)}$; (d) the scale reading of the culture medium containing cells using the filtered supernatant of the cell culture medium as

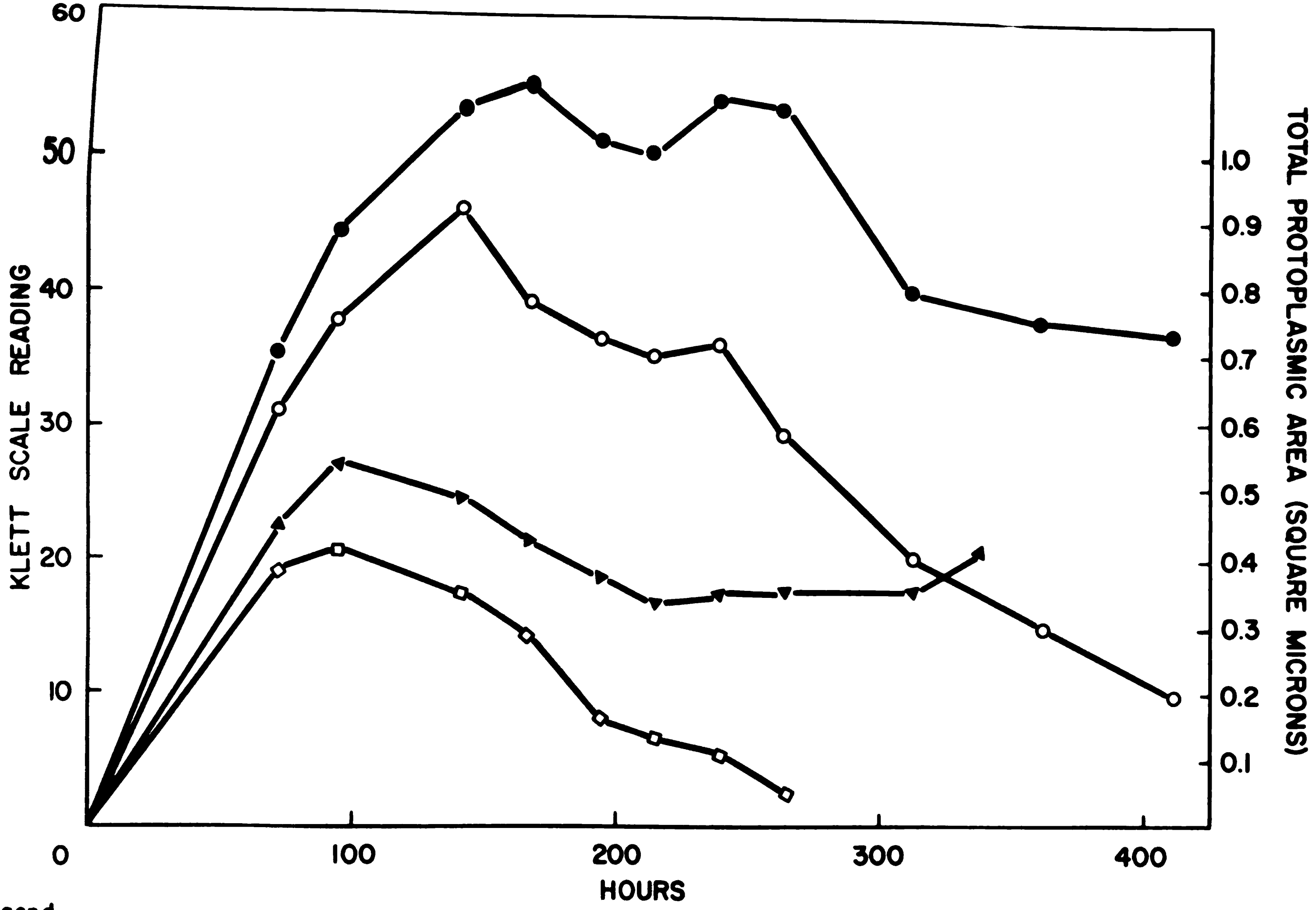
TABLE VIII

AN INTERPRETATION OF SOME OF THE DATA FROM TABLE VII

Total Hours	Bactone Media					Bactone Media Enriched With Thiamine				
	Cells/mm^3 (A)	Scale Reading (B)	Area per mm^3 (C)	$\frac{A}{B}$	$\frac{B}{C}$	Cells/mm^3 (A)	Scale Reading (B)	Area per mm^3 (C)	$\frac{A}{B}$	$\frac{B}{C}$
73	148	22	0.385	6.72	57.14	216	36	0.629	6.00	57.23
96	157	27	0.413	5.81	65.35	253	44	0.756	5.75	58.20
144	192	24	0.352	8.00	68.18	270	54	0.925	5.00	58.37
168	161	21	0.286	7.66	73.42	273	56	0.790	4.87	70.88
192	101	18	0.159	5.61	113.20	269	52	0.736	5.17	70.65
216	95	16	0.134	5.93	119.40	275	50	0.707	5.50	70.72
240	70	17	0.110	4.11	154.50	265	54	0.722	4.90	74.79
264	44	17	0.042	2.58	404.76	258	54	0.595	4.77	90.75
312	46	17	0.036	2.70	472.22	149	40	0.407	3.70	98.28
336	28	20	0.035	1.40	571.42					
360						130	38	0.299	3.42	127.09
408						72	37	0.196	1.94	188.77

FIGURE 2

Graphs of the total mean protoplasmic area and the Klett scale reading plotted against time.



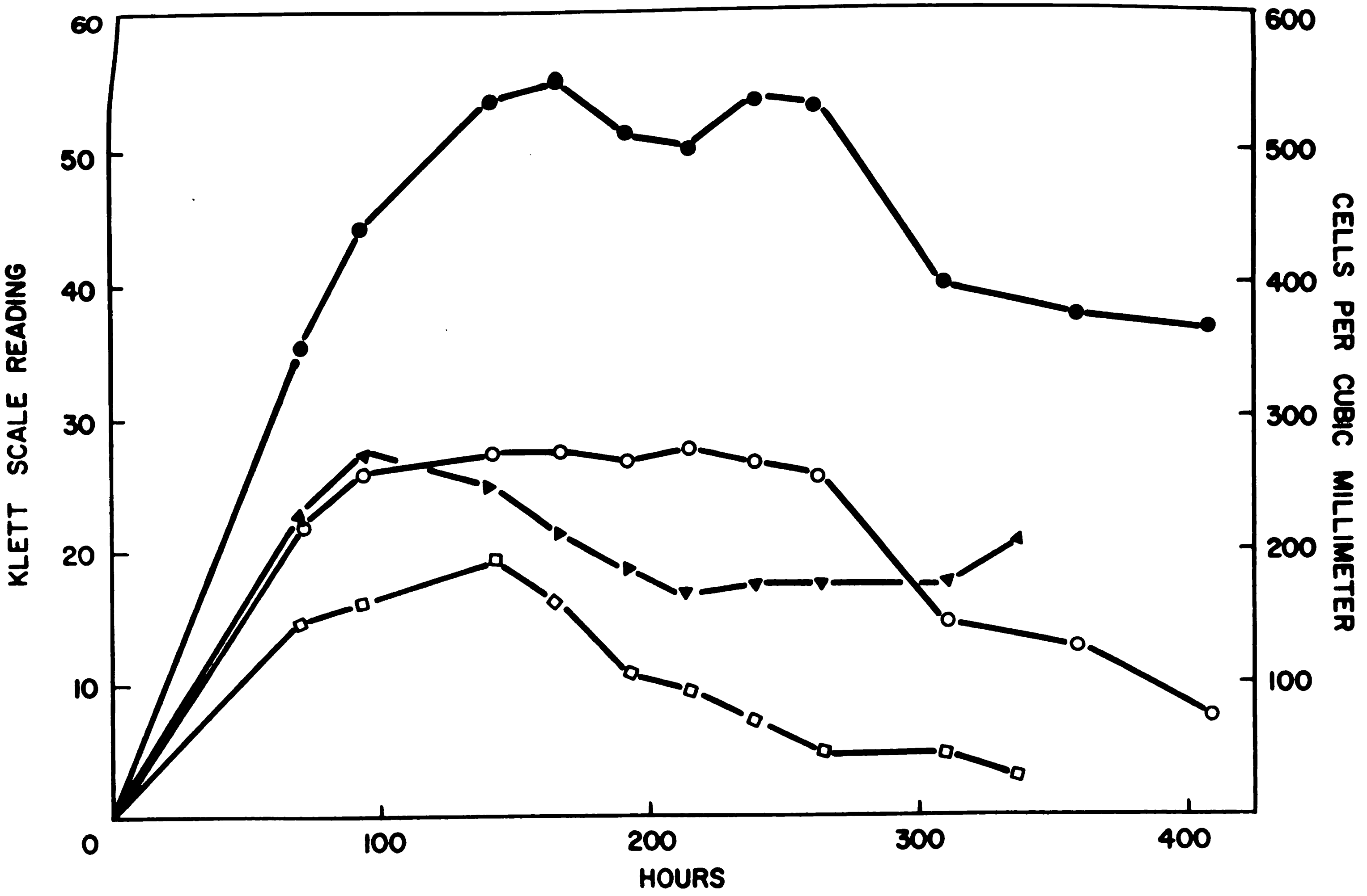
Legend

●-Klett Scale Reading; 1% enriched bactone medium; ▼-Klett Scale Reading; 1% bactone medium; and

○-total protoplasmic area; 1% enriched bactone medium; □-total protoplasmic area; 1% bactone medium.

FIGURE 3

Graphs of the number of cells per cubic millimeter and the Klett scale reading plotted against time.



Legend

●-Klett Scale Reading; 1% enriched bactone;

○-cells per cubic millimeter; 1% enriched bactone;

▼-Klett Scale Reading; 1% bactone medium; and

□-cells per cubic millimeter; 1% bactone medium.

the standard, $C^{(FS)}$; and (e) the scale reading of the filtered supernatant using fresh media as the standard, $FS^{(M)}$. All Klett scale readings were obtained using the blue filter. The results are summarized in Table IX. Figure 4 shows the rate of change in the optical density of the fractions described in (c) and (d) above.

Data presented in the preceding section indicated that the optical density was affected by factors other than cell count and total protoplasmic area. It was decided that an examination should be made of the optical density changes which might occur other than that due to the presence of cells. An examination of Table IX clearly indicates that the optical density of the filtered supernatant (FS), from all ages of bactone and enriched bactone cultures, does indeed change with time. For example, light absorption by materials in the supernatant accounted for approximately one half of the optical density of 72 hour bactone enriched cultures. Furthermore, this "light-absorbing factor" in the supernatant was brought about by the presence of the cells because as can be seen by a comparison of fresh media (M) with the filtered fresh media (FM), practically no change occurred in fresh media even though it had been exposed to the air for the same number of hours. At 168 hours after inoculation, the Klett scale reading for the non-enriched bactone culture medium, using fresh medium as the standard, $C^{(M)}$, was 58 and the scale reading of the supernatant, $FS^{(M)}$, was 22. Thus, the scale reading due to the cells alone, $C^{(FS)}$, was 36. Likewise, the maximal growth of the culture, $C^{(M)}$, appeared to occur at 168 hours in non-enriched bactone medium, but if the supernatant was used as the standard, $C^{(FS)}$, maximal growth occurred at 144 hours.

The previous discussion seems to indicate that in addition to the variables found in Part 1, namely, the cell count and total protoplasmic area, other factors can affect the optical density of the culture medium. An additional "light absorbing factor," which is present in the supernatant, must be taken into consideration if one wishes to use optical density measurements for estimating growth of protozoa. It appears that the best way to minimize the effect of the supernatant is to measure the optical density of the culture solution using the supernatant as the standard solution.

3. Klett scale readings obtained on the cell culture supernatant using blue, green and red filters.

This experiment was planned to see if perhaps a suitable filter, other than blue, might possibly negate the optical effect of the supernatant. A 2 per cent bactone medium enriched with thiamine (0.2 gamma per milliliter) was used for the culture medium. The data from this experiment is presented in Table X and Figures 5 and 6.

Dry weights. The determination of dry-weights was unsatisfactory. Table X shows a gradual increase in dry-weight which could be accounted for by the accumulation of dead cells. The dry-weight determination shows little correlation with either the direct cell count or the Klett scale reading. For example, at 72 hours there were 240 cells per cubic millimeter with a total dry weight of 0.56 milligrams per milliliter; at 144 hours there were 282 cells per cubic millimeter, and a dry-weight of 1.75 milligrams per milliliter. Thus, in 72 hours the number of cells per cubic millimeter increased by about 17 per cent and the dry-weight by over 200 per cent.

TABLE IX

KLETT SCALE READING TAKEN ON VARIOUS PORTIONS OF TWO DIFFERENT CULTURE MEDIA IN PART 2[a]

Total Hours	$C^{(M)}$		$C^{(FM)}$		$M^{(FM)}$		$C^{(FS)}$		$FS^{(M)}$	
	B	EB	B	EB	B	EB	B	EB	B	EB
72	0	23	0	24	0	-1	0	11	0	12
96	42	50	42	50	0	0	32	39	11	9
144	48	78	49	79	-1	+1	43	63	6	14
168	58	97	59	98	-1	-1	36	81	22	18
216	42	82	41	81	+1	-1	34	74	7	13
264	22	88	21	86	+1	-2	7	68	14	18

[a] Definitions of symbols used in Table IX:

B: One per cent bactone medium;

EB: one per cent bactone medium enriched with thiamine HCl (0.2 gamma/ml.);

C: culture medium containing cells;

M: fresh culture medium in which cells have never been grown;

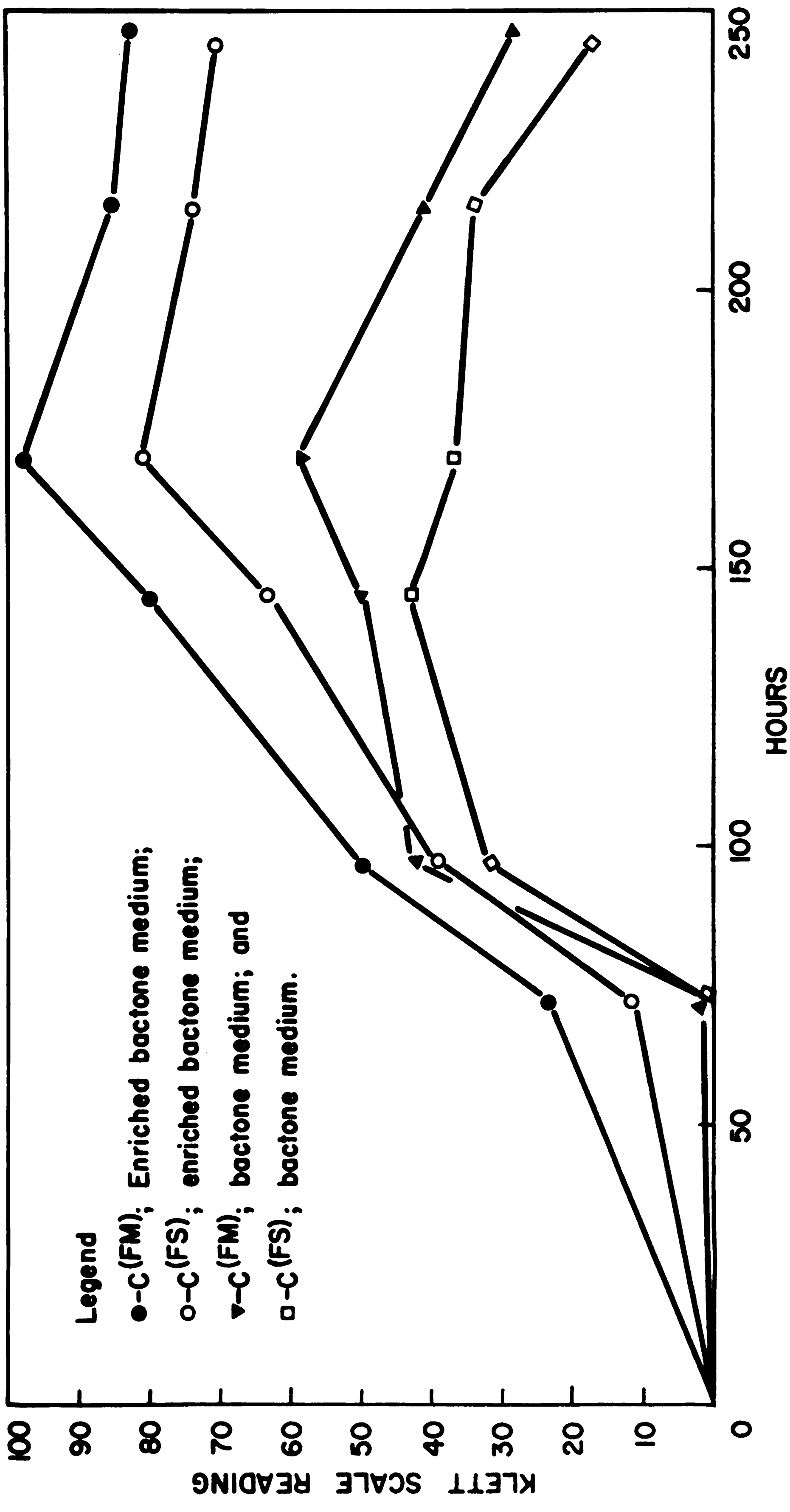
FM: filtered fresh culture medium in which cells have never been grown;

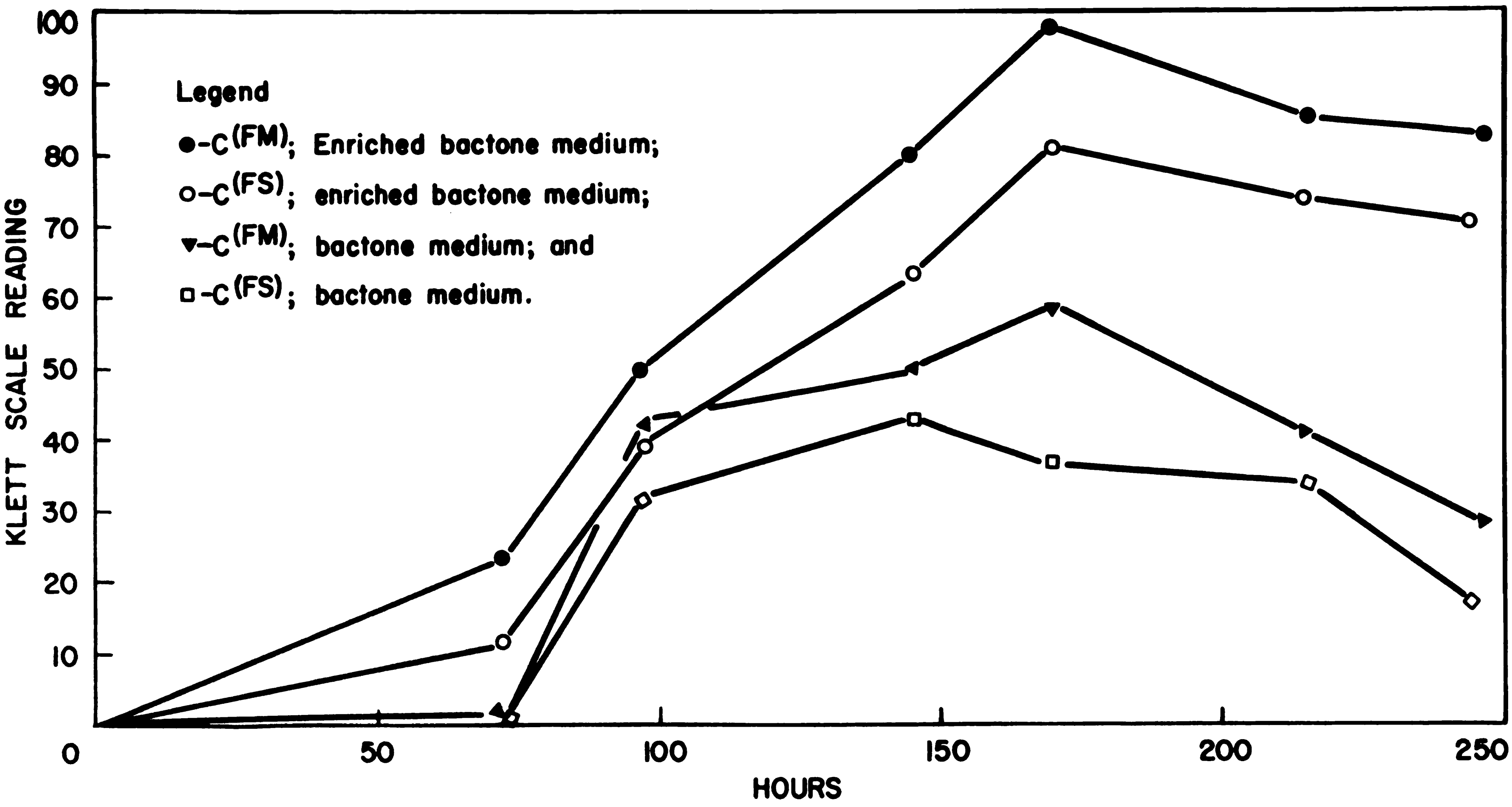
FS: filtered supernatant in which the cells have been growing; and

$C^{(M)}$: this symbol means that a Klett scale reading is taken on C (the culture medium containing cells) using M (fresh culture medium) as the standard solution. $C^{(FM)}$, $M^{(FM)}$, $C^{(FS)}$ and $FS^{(M)}$ were comparisons made in a similar manner.

FIGURE 4

Graphs of the Klett scale readings for cultures grown in a bactone medium and in an enriched bactone medium plotted against time. See footnotes to Table IX for definitions of symbols.


KLETT SCALE READING
100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
HOURS
Legend
●–C(FM); Enriched bactone medium;
○–C(FS); enriched bactone medium;
▼–C(FM); bactone medium; and
□–C(FS); bactone medium.


Legend
●-C(FM); Enriched bactone medium;
○-C(FS); enriched bactone medium;
▼-C(FM); bactone medium; and
□-C(FS); bactone medium.
KLETT SCALE READING
0
10
20
30
40
50
60
70
80
90
100
50
100
150
200
250
HOURS

Filters. The Klett scale readings suggest that: (a) the fresh culture medium was quite stable and if a change did occur it was one which produced a decrease in light absorption instead of an increase; (b) none of the three filters was selective for cells alone because all filters showed an increase in the optical density of the supernatant with time; and (c) at the beginning of the culture growth it appeared as though the supernatant had actually lost some optically absorbent material which was sensitive to the blue and green filter.

The maximum cell count of 282 cells per cubic millimeter was reached at 144 hours. At this time the scale reading (using the blue filter) of the culture, when fresh media was used as the standard, $C^{(M)}$, had increased to 66, while the scale reading of the supernatant, $S^{(M)}$, had increased to 18. Thus about 30 per cent of the reading, $C^{(M)}$, was due to the optical density of the supernatant. At the same time (144 hours) using the green filter it can be calculated that 5 per cent of the optical density of the cultre medium, $C^{(M)}$, was due to the optical density of the supernatant. Likewise, using the red filter, 20 per cent of the optical density of the culture, $C^{(M)}$, was due to the optical density of the supernatant.

It would seem from the observations cited above that a green filter provided the most accurate Klett scale reading for the measurement of growth.

4. The variation of the optical density of the supernatant with various types of media.

The following experiment was carried out as part of a plan for ascertaining whether or not the "light absorbing factor" in the

TABLE X

SUMMARY OF DATA COLLECTED FOR PART 3[a]

Total Hours	Direct Cell Count (Cells per mm^3)	Photometric Measurements					Dry Weight (mgms. per ml.)
		Filter	$M^{(B)}$	$C^{(M)}$	$S^{(M)}$	$C^{(M)} - S^{(M)}$	
24	2	Blue	183	0	-11	11	0
		Green	53	0	- 9	9	
		Red	7.5	4	2.5	1.5	
72	240	Blue	183	50	0	50	0.56
		Green	50	35	0	35	
		Red	7	28	2.5	25.5	
144	282	Blue	183	66	18	48	1.75
		Green	45	50	10	40	
		Red	4	37	8	29	
168	276	Blue	181	68	18	50	1.95
		Green	46	43	10	33	
		Red	6	30	8	22	
192	225	Blue	183	48	19	29	1.83
		Green	45	28	8	20	
		Red	6	22	8	14	
216	85	Blue	180	44	27	17	2.37
		Green	44	25	10	15	
		Red	6	15	5	10	
240	72	Blue	179	44	27	17	2.61
		Green	45	25	9.5	15.5	
		Red	5	14	6	8	

[a] Meaning of symbols used:

M: Fresh media in which cells have not been grown;
B: distilled water;
C: culture media containing cells;
S: supernatant, after centrifugation, of culture media in which cells have been grown; and
$M^{(B)}$: Klett scale reading of fresh media using distilled water as a standard.

FIGURE 5

Graphs of the Klett scale readings of the culture solution using red, green and blue filters plotted against time. See footnotes to Table X for definitions of symbols.

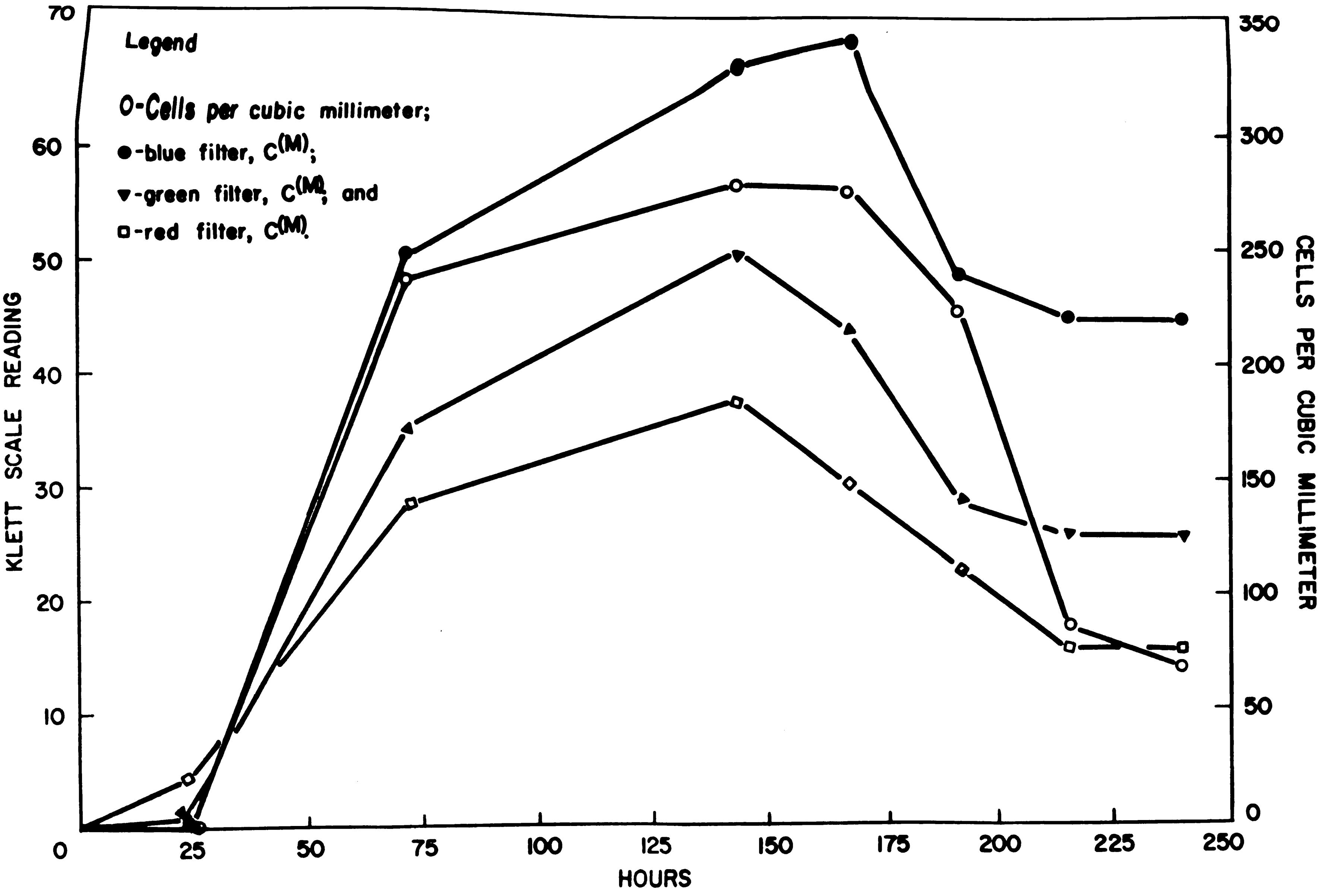
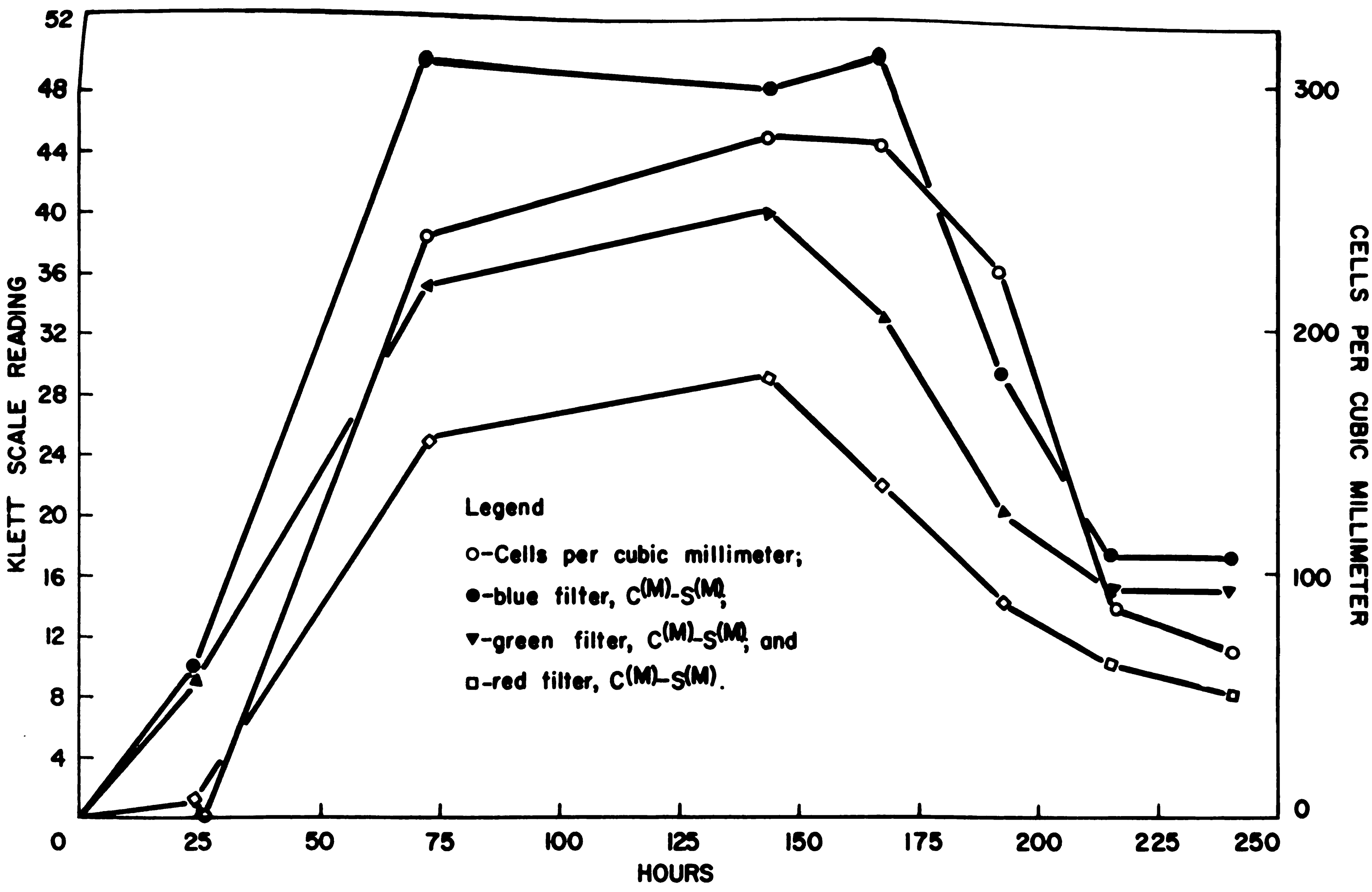

Legend
O-Cells per cubic millimeter;
●-blue filter, $C^{(M)}$;
▼-green filter, $C^{(M)}$; and
□-red filter, $C^{(M)}$.
KLETT SCALE READING
0
10
20
30
40
50
60
70
HOURS
25
50
75
100
125
150
175
200
225
250
CELLS PER CUBIC MILLIMETER
0
50
100
150
200
250
300
350

FIGURE 6

Graphs of the corrected Klett scale readings of the culture solution using red, green and blue filters plotted against time. See footnotes to Table X for definitions of symbols.


KLETT SCALE READING
52
48
44
40
36
32
28
24
20
16
12
8
4
0
25
50
75
100
125
150
175
200
225
250
HOURS
CELLS PER CUBIC MILLIMETER
300
200
100
0
Legend
○-Cells per cubic millimeter;
●-blue filter, C(M)-S(M);
▼-green filter, C(M)-S(M); and
□-red filter, C(M)-S(M).

supernatant was independent of or related to the constituents of the medium, e.g., proteoses, amino acids, etc. If this factor were found to be a constant and consistent quantity, then the scale readings could always be adjusted to give a more correct growth measurement. The data collected in this series of experiments were the following: (1) the Klett scale reading of the fresh media using distilled water as the standard, $M^{(B)}$; (2) the scale reading of the cell culture using fresh medium as the standard, $C^{(M)}$; (3) the scale reading of the supernatant using fresh media as the standard, $S^{(M)}$; and (4) the arithmetic difference between the scale reading for (2) minus the scale reading for (1), i.e., $C^{(M)} - S^{(M)}$. All Klett scale readings were taken using a blue filter. In this series of experiments the cell culture supernatant was not filtered because it was found that a clear supernatant could be obtained by centrifugation and a rapid drawing-off of the supernatant by way of a syringe (see "Materials and Methods").

Bactone medium. A 2 per cent bactone medium was used for the culture medium and the flasks were inoculated with cells which had been maintained in 2 per cent bactone medium for at least one year. The data for this experiment are summarized in Table XI and Figure 7.

It is interesting to note that 1-46 hours subsequent to inoculation of the cultures, the optical density was due to changes in the supernatant. Microscopic examination of the supernatant revealed neither cells nor particulate matter. It was also evident that the scale readings of the culture media, $C^{(M)}$, did not portray the usual type of growth curve. On the other hand, if the scale reading of the supernatant was subtracted from the scale reading of the culture medium,

one obtained a growth curve, as shown in Figure 7, which resembled the growth curve obtained by making direct cell counts. Figure 7 also shows that the graph of the optical densities of the supernatant is nearly a straight line, and possibly indicative of a direct proportionality of some kind. This is unusual in that one would expect the "light absorbing factor" in the supernatant to be related to the cell culture growth curve and thus should portray a somewhat logarithmic nature.

Bactone medium inoculated from a vitamin enriched culture. This experiment was essentially identical to the previous experiment. The same medium (2 per cent bactone) was used and the scale readings were obtained using the same filter. The cells used for inoculation had been maintained by Dr. R. A. Fennell of the Michigan State University Zoology Department, on a vitamin enriched bactone medium for nearly 12 months prior to this experiment. The question arose as to whether or not the transfer of organisms from a vitamin enriched bactone medium to a bactone medium would modify the metabolism of the protozoa and as a consequence alter the optical density of the supernatant. The data obtained in this experiment are given in Table XII and Figure 8.

Table XII shows essentially the same results found in the previous experiment except for a slightly enhanced growth curve which was probably attributable to the culturing of the organisms in the vitamin enriched medium prior to the transfer to the experimental solutions. It is evident in the table that for the first 24 hours, the optical density of the culture was essentially the same as the optical density of the supernatant. Thus the photometric readings appear to

TABLE XI

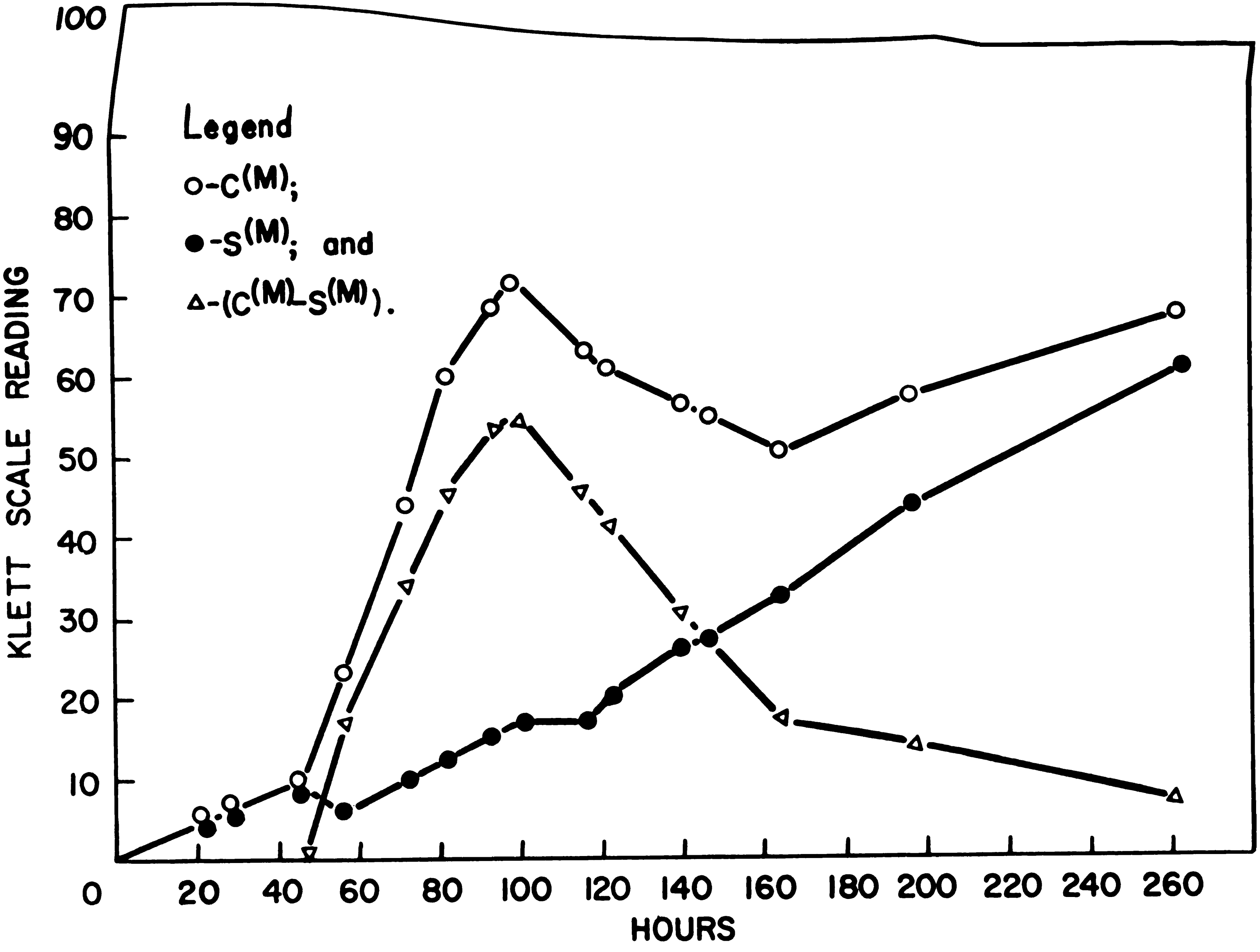
A SUMMARY OF KLETT SCALE READINGS TAKEN IN AN EXPERIMENT USING A TWO PER CENT BACTONE MEDIUM[a]

Age of Culture (Hours)	$M^{(B)}$	$C^{(M)}$	$S^{(M)}$	$C^{(M)} - S^{(M)}$
21	183	5	5	0
27	183	6	6	0
46	183	9	9	0
56	182	23	6	17
71	182	44	10	34
81	182	60	12	45
93	182	68	15	53
99	182	72	17	55
116	182	63	17	46
122	182	61	20	41
140	182	56	26	30
146	182	55	27	28
164	182	49	32	17
196	182	57	43	14
262	182	67	60	7

[a] See footnotes to Table X for the definitions of symbols $M^{(B)}$, $C^{(M)}$ and $S^{(M)}$.

FIGURE 7

Graphs of the Klett scale readings of the culture medium (bactone), the supernatant, and the numerical difference between these two measurements plotted against time. See footnotes to Table X for definitions of symbols.


Legend
○-$C^{(M)}$;
●-$S^{(M)}$; and
△-($C^{(M)}$-$S^{(M)}$).
KLETT SCALE READING
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
160
180
200
220
240
260
HOURS

be a measure of materials in the supernatant and not a measurement of growth of organisms. The second column in Table XII shows a relationship between optical density and age of the fresh bactone solution, i.e., as the age of the fresh medium increased there was a tendency for the optical density to decrease. It is evident in column four that the optical density of the supernatant increased from about 9 in 12 hour cultures to about 73 in 328 hour cultures. The graph of the scale readings of the supernatant, $S^{(M)}$, again appears to be a straight line function of some unknown factor.

Bactone medium "E". The highly nutritional medium designated as bactone medium "E" (see "Materials and Methods," Part A) was used in this experiment. This medium is well buffered in addition to the added growth factors present in yeast extract. In this experiment, as well as in all previous experiments, the flasks were inoculated from a stock culture with a bacteriological transfer loop. In all experiments, the final concentration after inoculation came to about one cell per cubic millimeter. The data are presented in Table XIII and the graphs of the various scale readings are given in Figure 9.

One of the most striking differences between this experiment and those cited in earlier sections was the lack of an increase in the optical density of the supernatant prior to cellular accumulation. Although the maximum growth was much higher than for a non-enriched 2 per cent bactone medium, the lag phase in the growth curve was very similar as can be seen in Figure 9. The increase in the scale reading of the supernatant was obvious but, surprisingly, was a somewhat slower increase even though the total growth was over three times as

TABLE XII

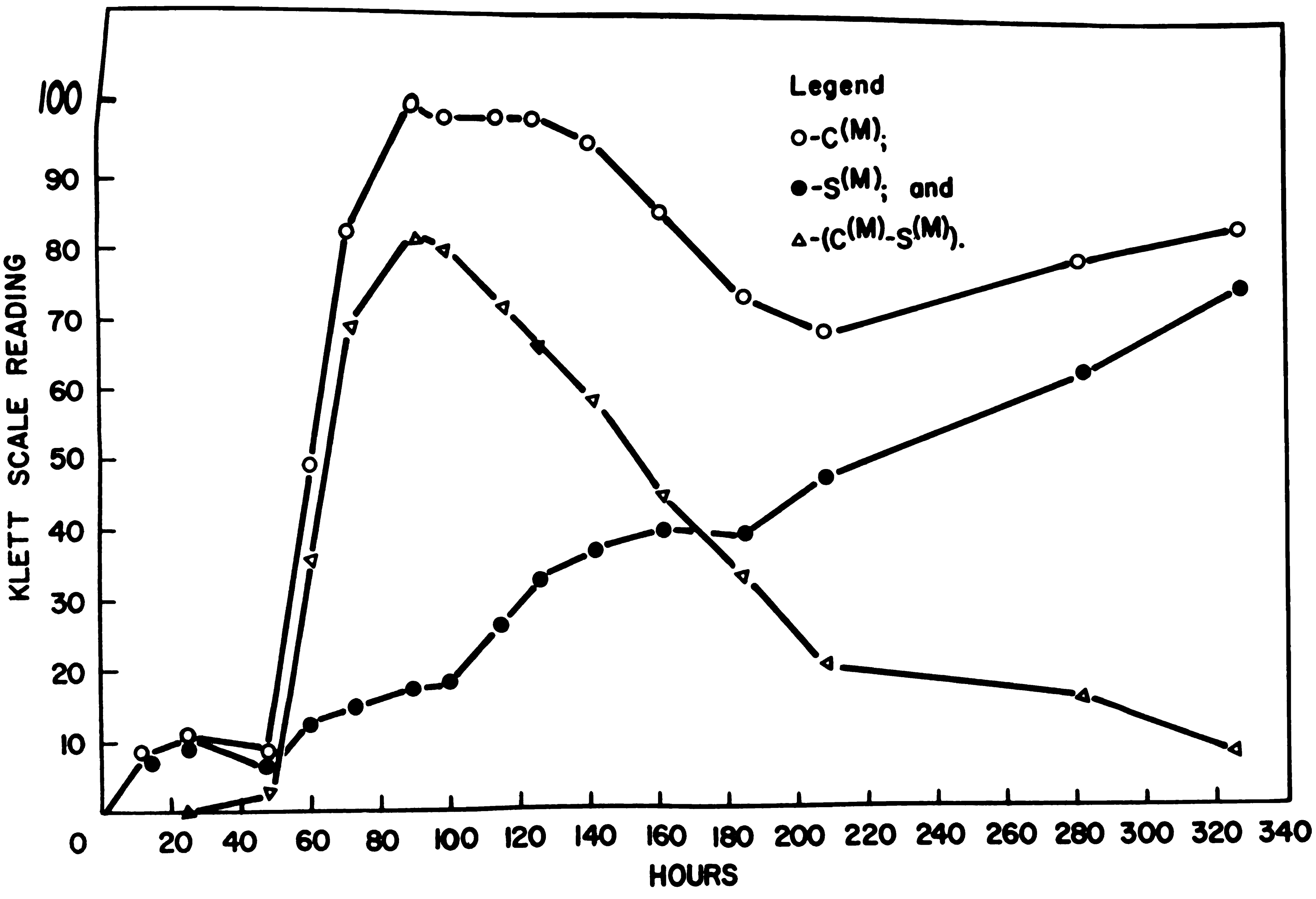
A SUMMARY OF KLETT SCALE READINGS TAKEN IN AN EXPERIMENT USING A TWO PER CENT BACTONE MEDIUM[a]

Age of Culture (Hours)	$M^{(B)}$	$C^{(M)}$	$S^{(M)}$	$C^{(M)} - S^{(M)}$
12	176	9	9	0
25	176	11	11	0
48	176	9	6	3
60	176	49	13	36
73	176	84	15	69
91	176	100	18	82
100	176	98	18	80
116	172	98	26	72
125	170	98	33	65
143	170	95	37	58
161	170	85	40	45
185	170	72	39	33
209	169	67	47	20
282	169	77	61	16
328	169	81	73	8

[a] See footnotes to Table X for the definitions of the symbols $M^{(B)}$, $C^{(M)}$ and $S^{(M)}$.

FIGURE 8

Graphs of the Klett scale readings of the culture medium (bactone), the supernatant and the numerical difference between these two measurements plotted against time. See footnote to Table X for definitions of symbols.


Legend
○-C$^{(M)}$;
●-S$^{(M)}$; and
△-(C$^{(M)}$-S$^{(M)}$).
KLETT SCALE READING
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
HOURS

great in bactone medium "E". For example, from Table XIII it can be seen that at maximal growth the Klett scale reading for the culture, $C^{(M)}$, and for the supernatant, $S^{(M)}$, was 348 and 17, respectively. Table XII shows a Klett scale reading for maximal growth of 100 for the culture, $C^{(M)}$, and 18 for the supernatant, $S^{(M)}$. It is interesting to note that although the maximal growth of the enriched bactone culture was over three times that in the non-enriched culture, the optical density of the supernatant in each case was about the same.

Bactone medium supplemented with $MgCl_2$. This experiment was undertaken to see if $MgCl_2$ would affect the nutritional balance in any way which could be detected by a photometric analysis. The medium used was a 2 per cent bactone medium enriched with $MgCl_2$ (1.0 milligram per milliliter). The same type of measurements were made as were obtained in the previous three experiments. The results are summarized in Table XIV.

The magnesium seemed to produce no particular effect. The scale readings were essentially similar to results obtained in 2 per cent bactone medium. In both cases, comparing Table XIV to Table XII, the maximal scale reading of the culture, $C^{(M)}$, was reached at about 90 hours subsequent to inoculation and at this time the scale readings of the supernatant, $S^{(M)}$, were nearly equal.

Chemically defined medium. This experiment utilized a chemically defined medium (see "Materials and Methods," Part B, Elliott's Medium). The data are summarized in Table XV and Figure 10 shows the graphs of the scale readings taken on the culture medium and the supernatant as well as a graph of the difference between these two

TABLE XIII

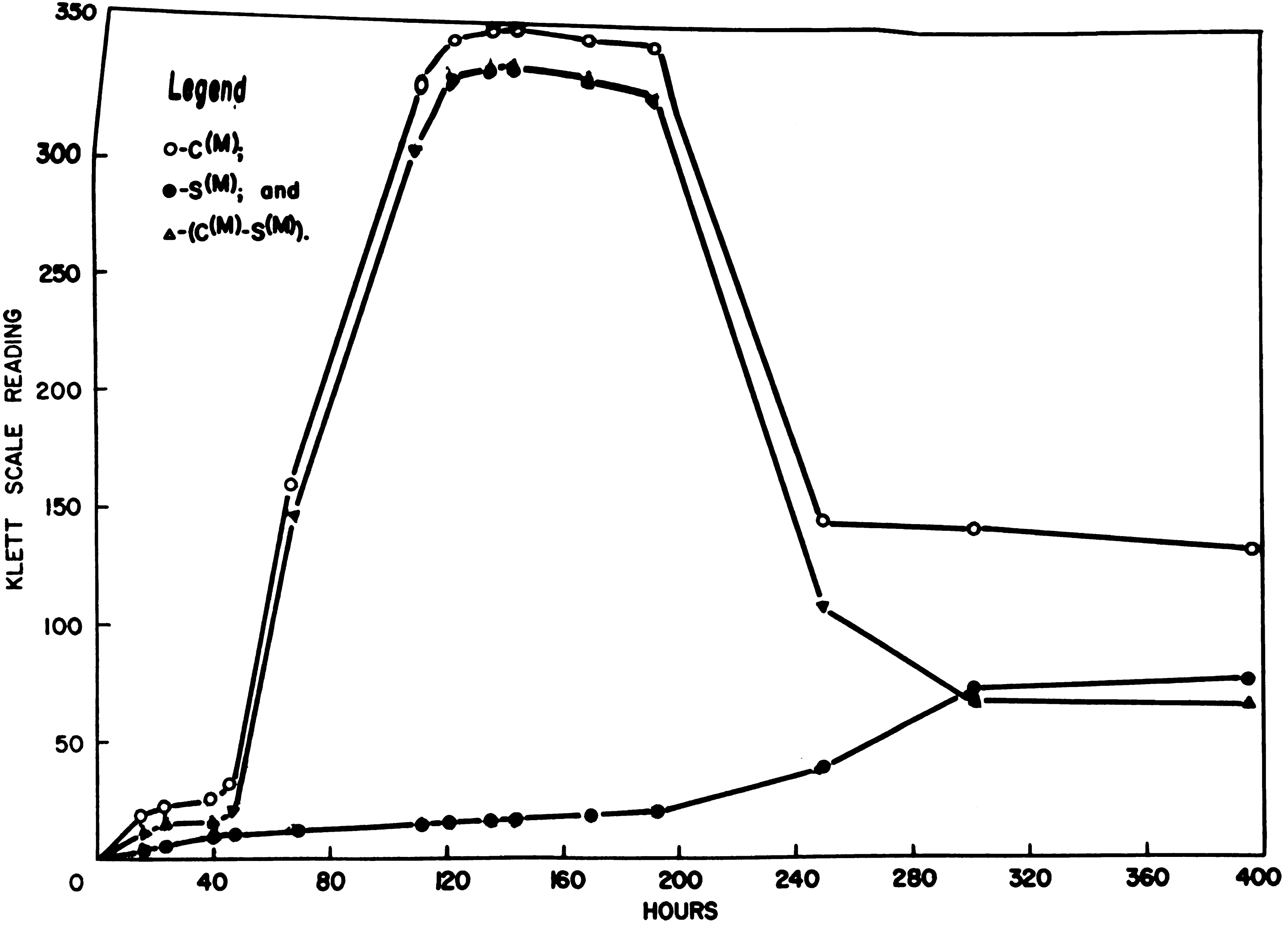
A SUMMARY OF THE KLETT SCALE READINGS TAKEN IN AN EXPERIMENT USING BACTONE MEDIUM "E"[a]

Total Hours	$M^{(B)}$	$C^{(M)}$	$S^{(M)}$	$C^{(M)} - S^{(M)}$
16	140	19	5	14
24	141	24	7	17
41	140	27	11	16
48	139	30	9	21
67	139	159	11	148
112	139	325	15	310
121	139	342	16	326
136	139	347	17	330
144	139	348	17	331
170	139	346	18	328
193	139	345	21	324
249	139	144	38	106
301	139	141	72	69
397	139	131	76	65

[a] See footnotes to Table X for the definitions of symbols $M^{(B)}$, $C^{(M)}$ and $S^{(M)}$.

FIGURE 9

Graphs of the Klett scale readings of the culture medium (bactone medium "E"), the supernatant and the numerical difference between these two measurements plotted against time. See footnotes to Table X for the definitions of symbols.


Legend
○-C(M);
●-S(M); and
△-(C(M)-S(M)).
KLETT SCALE READING
0
50
100
150
200
250
300
350
40
80
120
160
200
240
280
320
360
400
HOURS

readings. Table XVI shows an interpretation of some of the data in Table XV. A direct cell count was made using the Sedgewick-Rafter counting chamber.

Table XV, column two, shows that the chemically defined medium was stable. The optical density was very low compared to the bactone medium because only the essential metabolites were included in the chemically defined medium. Also, the large molecules of the proteoses and peptones present in the bactone medium are not present, which would probably account for the lower optical density. In this experiment we do not find the supernatant accumulating optical density as rapidly as in the bactone medium. For example, Table XV shows that at 210 hours the scale reading of the supernatant, $S^{(M)}$, was only about 17, whereas the scale reading of the supernatant, $S^{(M)}$, of a 2 per cent bactone culture (Table XII) at 209 hours was 67. In fact, the chemically defined medium was the only culture medium used so far in which the culture medium showed a scale reading before one was found for the supernatant. The graph of the rate of change for the scale reading of the supernatant found in Figure 10 again reveals a possible straight line function. When this graph is compared to the other supernatant graphs, as is done in Figure 11, one can see that the optical density of the supernatant of the chemically defined medium is considerably less than when the bactone media were used. Figure 11 also shows that although bactone medium "E" produced the greatest cell growth, the supernatant of bactone medium "E" had the smallest optical density number of all the bactone media used. Table XVI shows the calculations made in order to see if a proportionality constant could be found

TABLE XIV

A SUMMARY OF THE KLETT SCALE READINGS TAKEN IN AN EXPERIMENT USING A TWO PER CENT BACTONE MEDIUM ENRICHED WITH $MgCl_2$ (1.0 mg. per ml.)[a]

Age of Culture (Hours)	$M^{(B)}$	$C^{(M)}$	$S^{(M)}$	$C^{(M)} - S^{(M)}$
12	182	4	4	0
25	182	7	7	0
48	181	16	8	8
60	181	29	5	24
73	181	62	10	52
91	181	90	16	74
100	181	84	17	67
116	181	92	25	67
125	179	84	25	59
143	178	80	30	50
161	178	66	27	39
185	177	68	36	32
209	176	62	35	27
281	176	61	45	16
449	176	78	69	9

[a] See footnotes to Table X for the definitions of the symbols $M^{(B)}$, $C^{(M)}$ and $S^{(M)}$.

TABLE XV

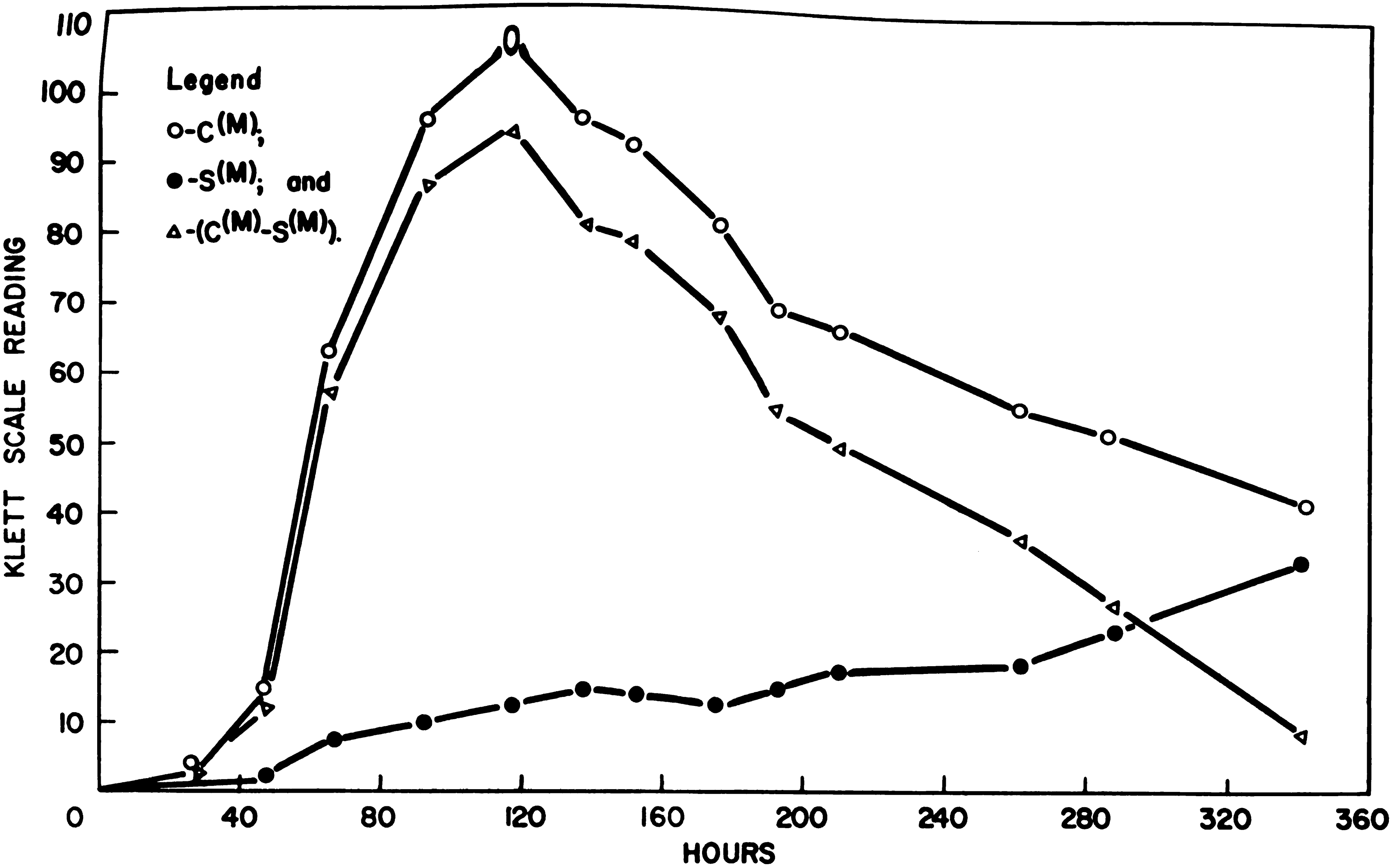
A SUMMARY OF KLETT SCALE READINGS OBTAINED IN AN EXPERIMENT USING A CHEMICALLY DEFINED MEDIUM (ELLIOTT)[a]

Age of Culture (Hours)	$M^{(B)}$	$C^{(M)}$	$S^{(M)}$	$(C^{(M)} - S^{(M)})$	Cells Per mm^3
15	2	0	0	0	2
27	2	3	0	3	6
48	2	14	2	12	82
67	2	64	7	57	191
93	2	97	10	87	212
118	2	107	12	95	228
138	2	96	15	81	188
152	2	93	14	79	180
176	2	81	13	68	178
193	2	69	15	54	152
210	2	66	17	49	108
262	2	54	18	36	92
287	2	51	24	27	73
341	2	41	32	9	36

[a] See footnotes to Table X for the definitions of the symbols $M^{(B)}$, $C^{(M)}$ and $S^{(M)}$.

FIGURE 10

Graphs of the Klett scale readings of the culture medium (chemically defined medium), the supernatant and the numerical difference between these two measurements plotted against time. See footnotes to Table X for the definitions of symbols.



Legend
○-$C^{(M)}$;
●-$S^{(M)}$; and
△-($C^{(M)}$-$S^{(M)}$).
KLETT SCALE READING
110
100
90
80
70
60
50
40
30
20
10
0
40
80
120
160
200
240
280
320
360
HOURS

TABLE XVI

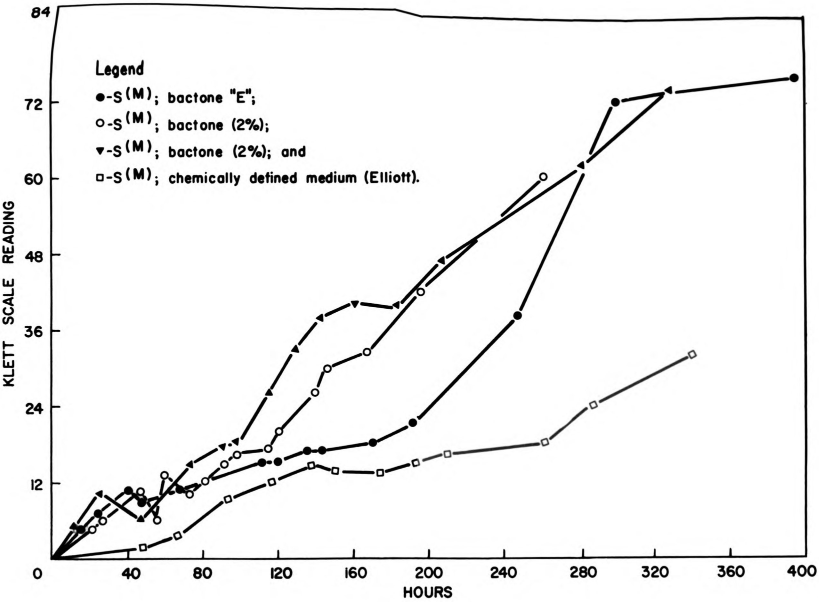
AN INTERPRETATION OF SOME OF THE DATA FROM TABLE XV[a]

Cells Per mm^3	$C^{(M)}$	$(C^{(M)} - S^{(M)})$	$\frac{\text{Cells Per } mm^3}{C^{(M)}}$	$\frac{\text{Cells Per } mm^3}{(C^{(M)} - S^{(M)})}$
82	14	12	5.86	6.8
191	64	57	2.98	3.36
212	97	87	2.18	2.42
228	107	95	2.13	2.40
188	96	81	1.96	2.32
180	93	79	1.94	2.28
178	81	68	2.20	2.62
152	69	54	2.20	2.82
108	66	49	1.64	2.20
92	54	36	1.70	2.56
73	51	27	1.43	2.70
36	41	9	0.88	4.00

[a] See footnotes to Table IX for the definitions of the symbols $C^{(M)}$ and $S^{(M)}$.

FIGURE 11

Graphs of the Klett scale readings of several types of culture supernatants, using fresh medium as the standard solution, plotted against time. See footnotes to Table X for the definition of the symbol $S^{(M)}$.

Legend
●-S(M); bactone "E";
○-S(M); bactone (2%);
▼-S(M); bactone (2%); and
□-S(M); chemically defined medium (Elliott).
KLETT SCALE READING
0
12
24
36
48
60
72
84
HOURS
40
80
120
160
200
240
280
320
360
400

between any of the data in Table XV. It is fairly obvious that not only does the chemically defined medium produce the least optically active supernatant, but that also the proportionality between the direct cell count and the corrected scale reading of the cell culture (by subtracting the scale reading of the supernatant) is quite good.

B. STUDIES ON CARBON DIOXIDE REQUIREMENT IN TETRAHYMENA

In order to facilitate the presentation of the data for the experiments in this section, the symbols listed and defined in Table XVII will be employed.

1. Aeration flask experiments.

The apparatus pictured in Plate 1 was used for this series of experiments. Chemically defined medium (see "Materials and Methods," Part B, Elliott's Medium minus glucose and sodium acetate) and bactone medium E minus glucose and sodium acetate were used as the culture media. The culture tubes containing 30 milliliters of medium were inoculated with one loop of a five day old culture of the same type of medium. This inoculation amounts to about 500 cells per milliliter in the culture tube at the commencement of the experiment. The objective of this experiment was to see if the removal of carbon dioxide from the air which entered the culture tube would inhibit the growth rate. The results are tabulated in Table XVIII. A direct cell count was made using the Sedgewick-Rafter chamber.

The data in Table XVIII did not reveal any consistent results which could be interpreted as indicating a carbon dioxide requirement for growth in Tetrahymena. In fact the results show that the growth in CO_2-free air was at least as good and in some cases better than in the control tubes. The growth of protozoa using the bactone medium did show a reduced rate when CO_2-free air was used in three out of the four trials but this decrease was not a significant amount. For reasons which will be stated in the Discussion this experimental set up was not considered a very satisfactory approach to the problem of the carbon dioxide requirement for Tetrahymena.

2. Desiccator-depression slide experiments.

The apparatus, described in "Materials and Methods," consisted of a sealed desiccator in which vessels of KOH had been placed in order to remove the carbon dioxide. Organisms were cultured in depression slides in both bactone (B'), and a chemically defined medium (Sy'). The control for this experiment was a desiccator containing vessels of 20 per cent KCl in place of the vessels of 20 per cent KOH. The volume of the culture was about 0.5 millileters, and the inoculation was such that the initial concentration of organisms was about 5 cells per culture drop. The control for this experiment was a desiccator containing vessels of 20 per cent KCl in place of the vessels of 20 per cent KOH. The data for this experiment are summarized in Table XVIII.

The number of cells present in both the control and the experimental cultures was essentially the same. There is no indication, at any rate, that the removal of carbon dioxide by absorption with KOH inhibited the growth of the culture.

TABLE XVII

SYMBOLS EMPLOYED FOR SECTION B

Symbol	Definition
$\overline{Ac}$	Acetate radicle ($C_2H_3O_2^-$);
B	bactone media E (see "Materials and Methods," Part A);
B'	bactone media E minus glucose and sodium acetate;
m	molality, i.e., number of moles dissolved in 1000 grams of solvent;
M	molarity, i.e., number of moles dissolved in 1000 milliliters of solvent;
Sy	chemically defined medium (see "Materials and Methods," Part B, Elliott's medium);
Sy'	chemically defined medium (Elliott) minus glucose and sodium acetate; and
T	Trial or sample number of the culture used in a given experiment.

TABLE XVIII

RESULTS OF EXPERIMENTS IN PARTS 1, 2 AND 3 WHICH INVOLVED VARIOUS METHODS USED IN AN ATTEMPT TO REMOVE CARBON DIOXIDE FROM THE ENVIRONMENT OF THE CELLS

Description of Method	Trial Number	Hours Growth	Control		Experimental	
			B'	Sy'	B'	Sy'
Aeration Flasks	1	92	63[a]	138[a]	77[a]	126[a]
	2	75	81	122	43	186
	3	80	90	133	87	142
	4	82	128	118	98	113
Desiccator-Depression Slide Apparatus	1	72	850[b]	680[b]	700[b]	740[b]
	2	80	600	630	510	470
	3	76	580	700	630	730
Petri dish-Depression Slide Apparatus	1	68	340[b]	450[b]	480[b]	420[b]
	2	72	550	300	720	640
	3	72	800	600	420	750

[a] Cells per cubic millimeter.

[b] Cells per culture.

3. Petri dish-depression slide experiments.

This experiment was set up using the apparatus described in "Materials and Methods" in which a depression slide was placed on top of a stack of six slides in a Petri dish. The Petri dish was nearly filled with a 20 per cent KOH solution and a drop (about 0.5 milliliters) of inoculated medium was placed in the depression of the top slide. Both types of media, as used in the previous two experiments, were employed. The initial concentration of the drop culture was about 5 cells per culture. The control for this experiment involved the use of Petri dishes filled with 20 per cent KCl instead of KOH. The results are tabulated in Table XVIII.

This type of experiment did not produce any difference in growth rate, in experimental, and control solutions. The number of organisms in the culture were highly variable. Several additional attempts at this type of experiment resulted in bacterial and fungal contaminations. The fact that contamination occurred seems to indicate that the carbon dioxide was not adequately removed from the environment because many bacteria and molds are very sensitive to low carbon dioxide tensions. The very nature of the experiments in Parts 2 and 3 were such that it was difficult to maintain sterile conditions.

4. Moist chamber experiments.

This experiment is summarized in Table XIX. A depression slide was used in this experiment and a small drop of inoculated culture medium containing only one cell was placed within the depression. Near the culture drop but not touching it was placed a small drop of

0.3 molal KOH (experimental) or 0.31 molal KCl (control). A ring of vaseline was placed around the depression and a cover glass sealed to the vaseline, thus producing a sealed volume around the depression containing the two drops. The same media as used in Part 3, namely B' and Sy', were used.

One can see from the results in Table XIX that the experimental cultures grew much better than the controls for at maximum growth there were over four times as many cells in the depression slides which contained the KOH than in the control slides. It is also interesting to note that the lag phase of the growth curve was somewhat longer for the experimental culture than for the control.

5. Van Tieghem chamber experiments.

Experiments which utilized Van Tieghem chambers are summarized in Table XX. A bactone medium (B) was used for the culture medium. Three drops of moistener were used for each chamber. Varying concentrations of KOH were used for the experimental cultures and three drops of distilled water were used as a moistener for the control cultures. Each culture drop contained four cells at the inception of the experiment. Table XX shows that the cultures were discontinued at various times. This was done in order to take the pH of the cultures. In all cases, using pHydrion papers (Micro Essential Laboratory, Brooklyn, New York), the pH was between 7.0 and 7.4. Figure 12 shows the graphs of the rate of change in cell count for the various concentrations of KOH used. Plate 5 shows several photomicrographs of live cells as they appear in Van Tieghem cultures.

TABLE XIX

NUMBER OF ORGANISMS PER CULTURE USING SEALED DEPRESSION SLIDES

Age of Culture (Hours)	0.31m KCl Moistener			0.3m KOH Moistener		
	T1	T2	T3	T1	T2	T3
0	1	1	1	1	1	1
24	4	2	3	2	2	1
72	27	31	29	10	7	12
96	34	47	41	32	13	20
144	81	76	83	121	22	76
168	86	92	64	341	108	202
192	72	83	37	430	241	372
240	34	53	27	521	467	422
288	13	17	11	411	370	360
408	6	4	3	42	312	260

TABLE XX

CELL COUNTS IN VAN TIEGHEM CHAMBERS USING VARIOUS CONCENTRATIONS OF KOH AS A MOISTENER

Age of Culture (Hours)	H_2O Moistener			KOH Moistener														
				0.1M			0.2M			0.3M			0.4M			0.5M		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
0	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
6	9	15	10	7	6	8	6	8	8	8	8	10	4	10	9	10	11	10
11	15	27	17	14	12	16	11	13	18	19	12	17	5	14	13	16	15	13
23	63	122	92	30	42	48	21	27	21	22	14	21	8	31	20	16	13	12
30	102	212	132	61	62	94	30	33	27	31	19	27	8	30	18	14	13	14
50	520	1200	1000	220	218	340	92	50	36	43	17	23	8	23	13	8	6[a]	8
77	1500	1500	1500	700	680	820	131	62	54	49	15	16	6	17	9	6	60	3[a]
96	1875	1750	1870	920	870	890	180	95	72	51	13	15	3[a]	15	6[a]	6	450	24
126	2000	1900	2000	900	1200	1000	520	360	104	47	9	15	10	16	18	6	1400	600
138	2000	2000	2000	900	1200	900	600	420	125	41	9	15	120	16	31	6[a]	1500	1200
163	1900	1800	1900	900	1200	900	600	415	150	38	9	15	600	16	65	6	1600	1800
190	1800	1800	1800	900	1200	900	650	400	182	36	9	15	1700	16	1200	9	1800	1800
258	1800	1800	1800	820	900	840	540	400	220	31	9	13	1700	16	1600	520	1800	1800
282	1800	1800	1800	680	800	620	520	400	232	28[a]	9	13	1900	16	1700	1300	1600	1800
306	1650		1200	680	800	620	500	390	270	31			1600		1650	1700	1400	1500
426	1600		950	680	780	600	480	380	341	142			1500		1300			
450	1250		850	650	750	600	440	460	460	400								
474							320	175	270									

[a] The KOH solution was replaced with distilled water at this point in the experiment.

One can see from Figure 12 that the number of cells per culture is inversely related to the molal concentration of the KOH. When distilled water was substituted for KOH (columns six and seven, Table XX) the number of organisms in individual cultures rapidly increased. This experiment was noteworthy in that it showed the great inhibition of growth induced by hypertonic solutions of KOH when they are used as moisteners in Van Tieghem chambers. In subsequent experiments, controls were established by the substitution of either an isosmotic solution of KCl or sodium acetate for KOH as a moistener in the Van Tieghem chamber. The pH measurements showed a variation of about one-half of a pH unit during the experiment. Thus the absorption of carbon dioxide by the KOH does not raise the pH to any extent.

Isosmotic moisteners. A series of experiments were performed in order to compare the growth of cells in Van Tieghem chambers in which isosmotic solutions of KCl, sodium acetate, and KOH were used as moisteners. Six drops of moistener were used in all culture chambers. The experimental chambers contained 0.3 molal KOH for the removal of carbon dioxide; the control chambers contained 0.3 molal sodium acetate or 0.31 molal KCl. The culture medium for this experiment was bactone medium "E" minus glucose and sodium acetate. The data are summarized in Tablx XXI and the graphs of the average cell counts for the three types of cultures are found in Figure 13.

Some of the observations that should be noted from a study of Table XXI and Figure 13 are: (a) the rate of growth when KOH is used as a moistener was much slower than in the controls, however the maximum growth in the "KOH cultures" ultimately exceeded the maximum growth

FIGURE 12

Graphs of the rate of change in the cell count using various concentrations of KOH as moisteners in the Van Tieghem chambers.

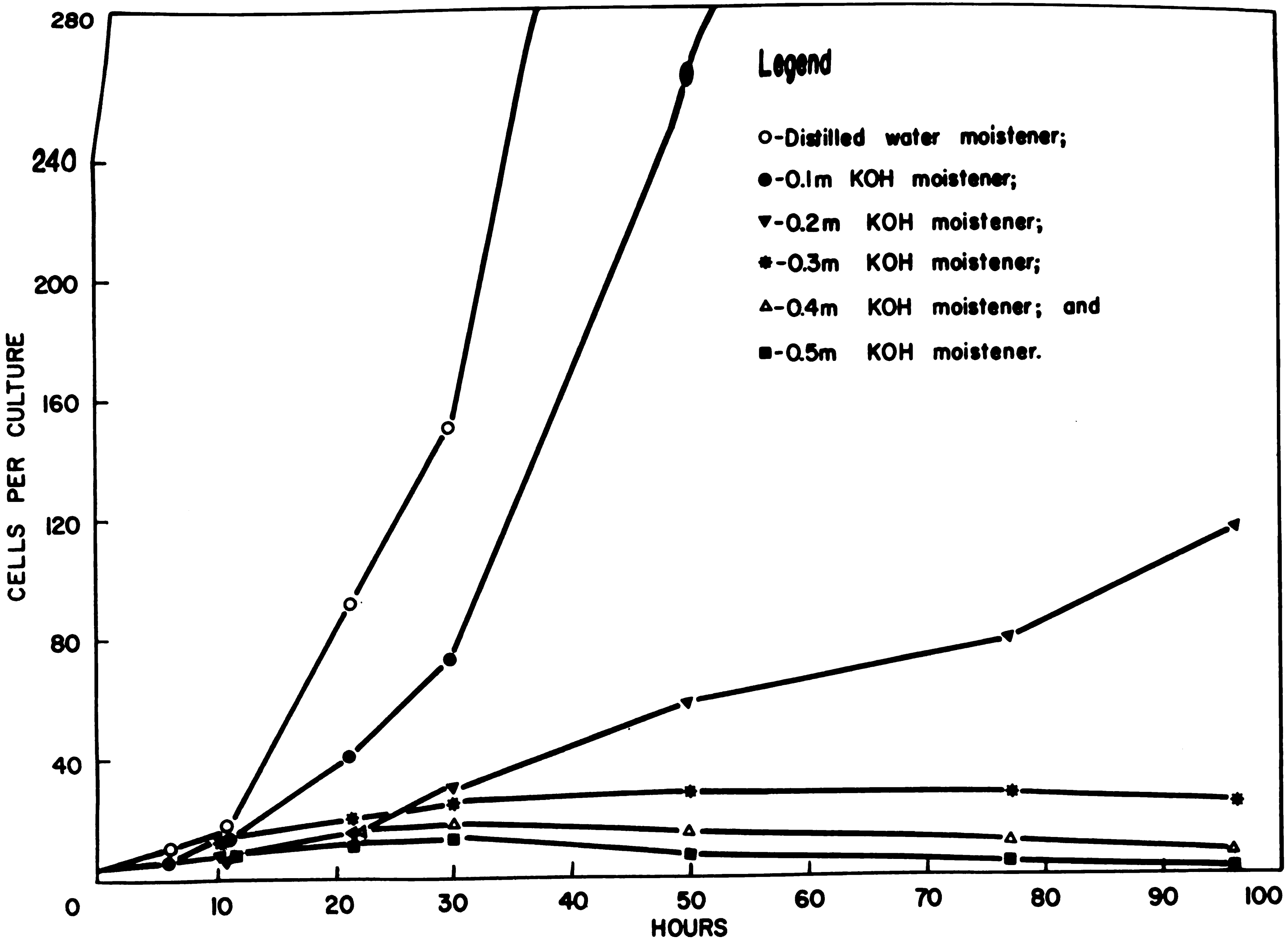
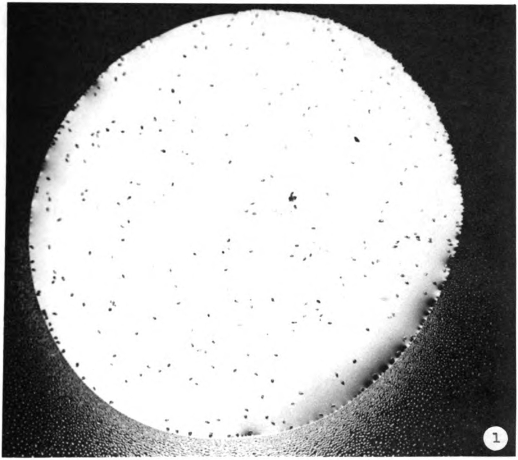

280
240
200
160
120
80
40
0
CELLS PER CULTURE
10
20
30
40
50
60
70
80
90
100
HOURS
Legend
○-Distilled water moistener;
●-0.1m KOH moistener;
▼-0.2m KOH moistener;
✱-0.3m KOH moistener;
△-0.4m KOH moistener; and
■-0.5m KOH moistener.

PLATE 5

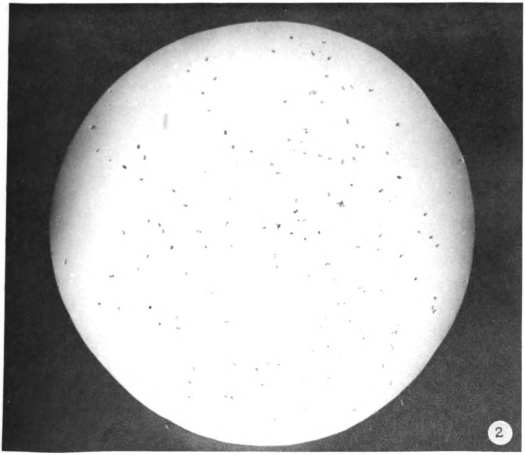
Photomicrographs of live cells as they appear in the culture drop of a Van Tieghem chamber.

Fig. 1. Distilled water moistener; and

Fig. 2. 0.2 molar KOH moistener.



①



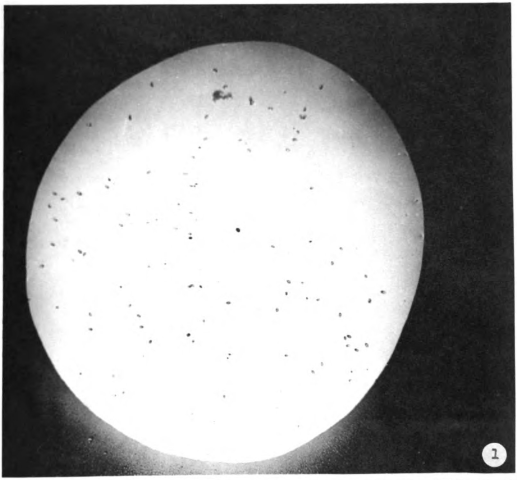
②

PLATE 6

Photomicrographs of live cells as they appear in the culture drop of a Van Tieghem chamber.

Fig. 1. 0.3 molar KOH moistener; and

Fig. 2. 0.5 molar KOH (note tyrosine crystal).


1

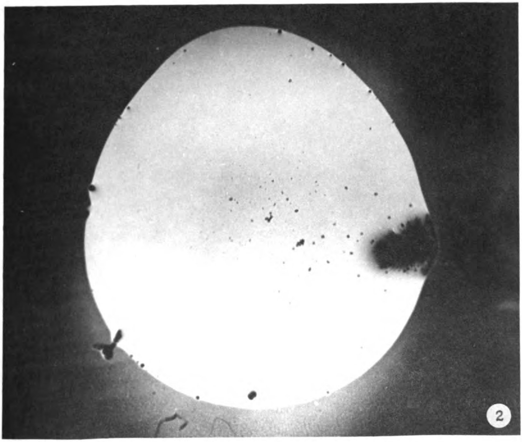
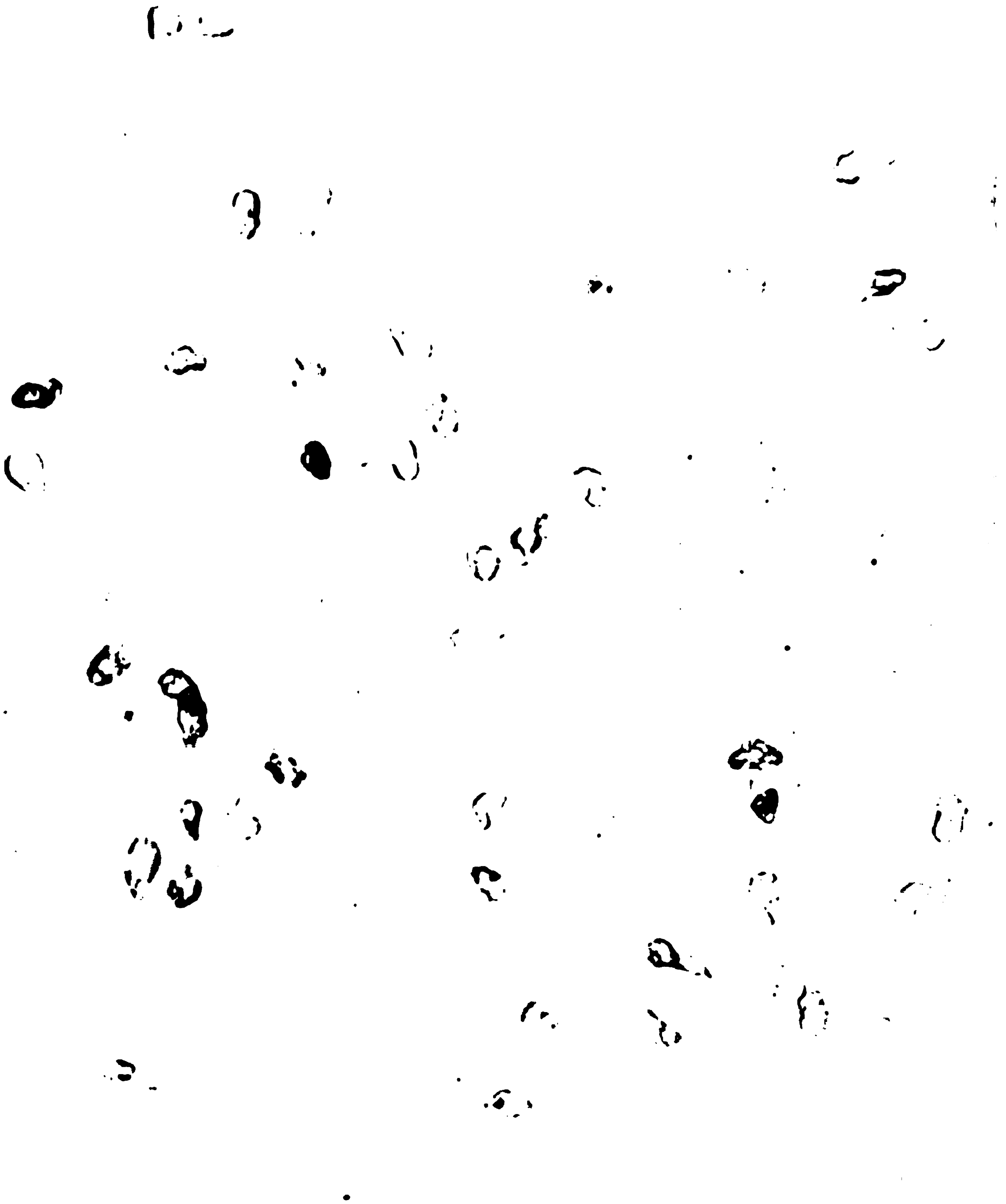

2

PLATE 7

Photomicrograph of live cells in the culture drop of a Van Tieghem chamber. The organisms are cultured in a bactone medium supplemented with 0.35 per cent agar.



of the controls (Figure 13); and (b) when sodium acetate was used as a moistener, the number of organisms decreased from a maximum in 200 hour cultures to zero in 340 hour cultures. It should be pointed out that the cytoplasm in the cells grown in "sodium acetate chambers" became extremely granular soon after the maximum cell count was reached. The pH of the culture solutions in the "sodium acetate chambers" decreased to about 5.5 when the number of organisms decreased to almost zero. The pH of the other cultures utilizing KOH or KCl remained fairly constant at about pH7.

Experiments testing pH constancy and moistener molalities.

(a) A series of Van Tieghem chambers were set up using both B' and Sy' as culture media and utilizing varying concentrations of KOH from 0.1 molal to 0.6 molal as moisteners. Six drops of the KOH solutions were used. The drops of uninoculated medium were left in the Van Tieghem chambers for about 200 hours. The pH, using pHydrion papers, of the medium was taken at the end of 200 hours.

In all cases the pH of the media remained constant. There was no evidence that the KOH "creeped" along the walls of the chamber to reach the culture solution. It was felt, therefore, that the pH was not a variable in these experiments.

(b) The following combinations of medium and moisteners for the Van Tieghem chambers were made:

(1) A 2 per cent bactone medium minus glucose and sodium acetate using 0.4 molal KOH as a moistener;

(2) a 4 per cent bactone medium minus glucose and sodium acetate using a 0.5 molal KOH as a moistener;

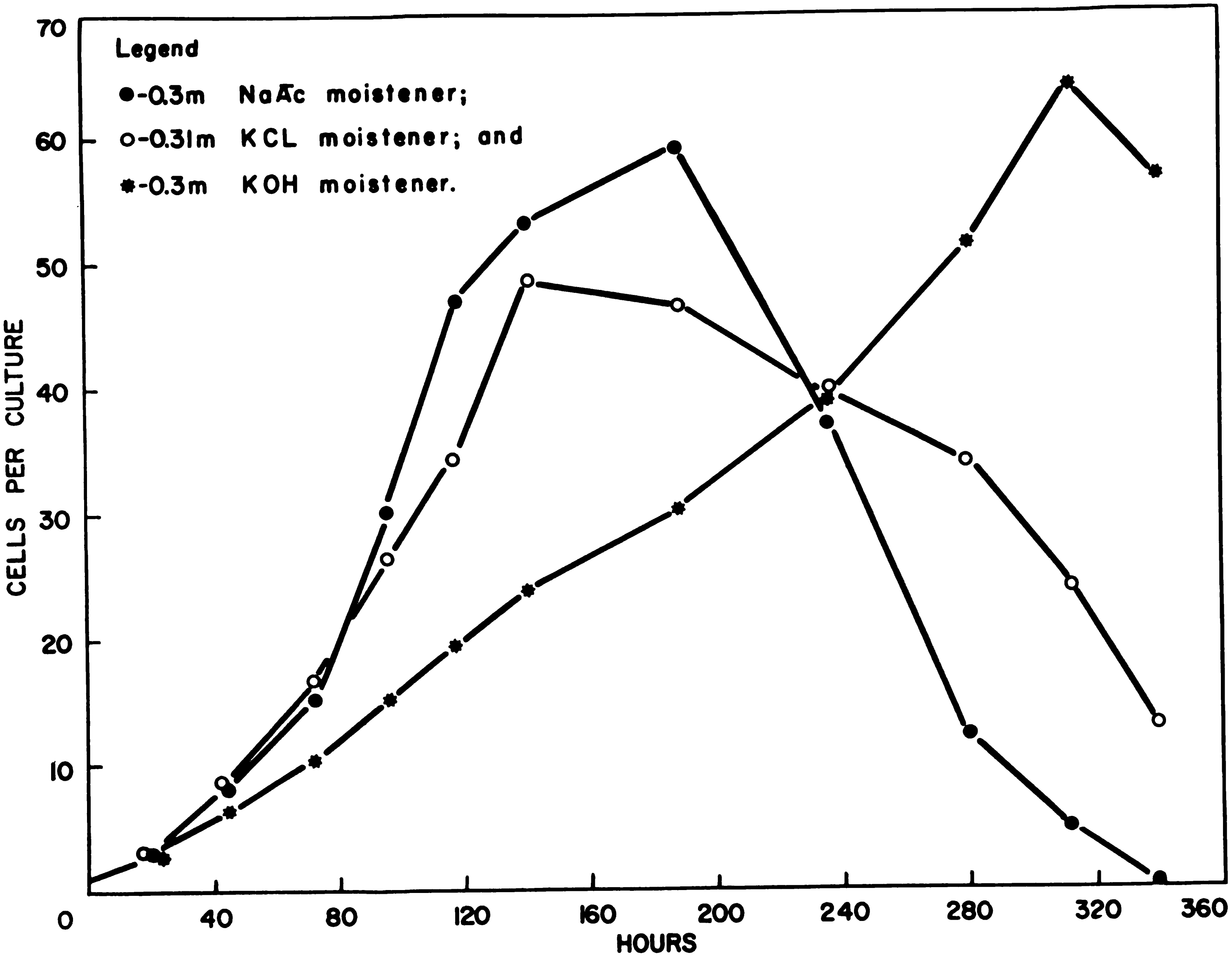
TABLE XXI

CELL COUNTS USING VAN TIEGHEM CHAMBERS CONTAINING 0.3m Na ACETATE, 0.31m KCl AND 0.3m KOH AS MOISTENERS

Age of Culture (Hours)	Type of Moistener Used in Van Tieghem Chamber											
	0.31m KCl				0.3m Na$\overline{\text{Ac}}$				0.3m KOH			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
0	1	1	1	1	1	1	1	1	1	1	1	1
20	3	4	4	2	4	2	4	3	4	2	3	4
44	7	8	9	9	6	8	8	9	5	6	4	7
73	17	12	19	15	13	15	17	16	11	9	10	8
96	24	28	31	21	29	33	27	32	15	16	14	18
117	35	36	38	29	50	49	42	47	17	21	15	23
141	49	52	47	46	57	53	49	55	19	24	17	31
189	51	50	43	41	65	60	62	51	22	31	21	46
237	37	48	40	33	23	43	45	37	40	38	28	51
280	36	41	31	30	10	13	17	12	53	47	43	60
312	22	36	17	21	3	7	6	4	62	67	58	69
340	17	12	14	8	0	0	0	0	52	61	55	57

FIGURE 13

Graphs of the rate of change in cell count using KOH, KCl or Na acetate in the Van Tieghem chambers.


Legend
●-0.3m NaĀc moistener;
○-0.31m KCL moistener; and
✱-0.3m KOH moistener.
CELLS PER CULTURE
70
60
50
40
30
20
10
0
40
80
120
160
200
240
280
320
360
HOURS

(3) a 5 per cent bactone medium minus glucose and sodium acetate using 0.4 molal KOH as a moistener; and

(4) a chemically defined medium (Elliott's minus glucose and sodium acetate) in which all amino acid concentrations were doubled and using 0.4 molal KOH as the moistener.

The purpose of the series listed in (1) through (4) above was to see if it were possible to increase the carbon dioxide absorbing capacity of the KOH solutions by increasing the molality of the KOH. Since the increased molality of the KOH requires an increased osmotic equivalent in the culture medium, an attempt was made to increase the osmotic activity of the medium by increasing its concentration of metabolites.

The results of these experiments were completely negative. No concentration in the bactone or chemically defined media could compete osmotically with 0.4 molal KOH. In all cases only a few cell divisions took place and after the second day the cells became inactive and resembled spherical "cysts."

(c) A series of Van Tieghem chambers were set up, as described in "Materials and Methods," in which 16 drops of moistener were used instead of just six drops. After placing 16 drops of moistener in the chamber, the cover glass (containing the inoculated culture drop) was placed on top of the glass ring. Under these conditions the culture drop was situated within the air space formed by the meniscus of the moistener. Both types of media, namely B' and Sy', were used for this experiment. The concentrations of KOH and KCl were 0.3 molal and 0.31 molal, respectively.

There was no significant difference between the results obtained using this method and those obtained when only six drops of moistener were used (Table XXI), i.e., the growth of the cultures using KOH as the moistener was slower at the early stages but ultimately reached a higher maximum. In fact, in many cases the "KOH cultures" not only reached a higher maximum cell count, but the high cell count remained constant over a longer period of time. It seemed possible from these experiments that perhaps not enough of the carbon dioxide was removed from the environment and that alternative methods must be devised for a more efficient removal of carbon dioxide.

Dubnoff shaking incubator with chemically defined medium.

The objective of these experiments was to find a more efficient method for removing carbon dioxide from the environment of the cell. Six drops of moistener were placed within the Van Tieghem chamber. A small piece of filter paper (8 by 12 millimeters) was inserted in a vertical position within the Van Tieghem chamber. These chambers were placed on a rack to which masking tape had been attached in such a way that the sticky side was facing upward. The rack was then placed in the Dubnoff Metabolic Shaking Incubator and the apparatus was set so as to produce about one complete oscillation per second. The sticky portion of the tape held the chambers firmly in place when the shaking apparatus was in operation. The chambers were positioned on the rack so that the plane of the filter paper within the Van Tieghem chamber was perpendicular to the direction of oscillation. Chemically defined medium (Sy') was used. The moisteners were 0.31 molal KCl (control) and 0.3 molal KOH (experimental). The data are presented in Table XXII and the growth curves are depicted in Figure 14.

TABLE XXII

CELL COUNTS USING VAN TIEGHEM CHAMBERS WHICH WERE PLACED IN THE DUBNOFF METABOLIC SHAKING INCUBATOR. CULTURE MEDIUM: Sy'.

Age of Culture (Hours)	Type of Moistener Used in the Van Tieghem Chamber									
	0.31m KCl					0.3m KOH				
	T1	T2	T3	T4	Av.	T1	T2	T3	T4	Av.
0	2	2	2	2	2	2	2	2	2	2
19	7	13	8	13	10	8	10	4	4	6
27	14	25	15	24	19	9	16	4	6	9
40	26	42	38	40	36	15	23	9	10	16
47	34	49	40	41	41	16	26	10	12	16
63	39	51	41	47	44	19	29	12	14	18
74	40	53	41	45	45	23	31	12	16	20
88	42	52	43	44	45	25	33	12	15	21
114	37	51	47	48	46	30	43	13	17	26
136	34	57	55	49	49	27	54	14	18	28
190	37	44	46	40	42	23	39	11	17	22
218	32	38	37	38	36	21	27	12	16	19

Figure 14 shows that a KCl moistener produced better growth than a KOH moistener under the conditions described above. Likewise, it is evident that, in addition to a longer lag phase, the KOH culture also possessed a lower maximum cell count than the control. The results of this experiment will be compared to subsequent experiments which involved the use of a bactone medium (B') instead of the chemically defined medium (Sy').

Dubnoff shaking incubator with bactone medium. These experiments are quite similar in plan to the previous experiment. The Dubnoff Metabolic Shaking Incubator was again used and the pieces of filter paper were inserted into the Van Tieghem chamber. The primary difference between the two experiments was that the bactone medium (B') was used for this experiment and sodium acetate (0.3 molal) was used as a control in addition to KCl (0.31 molal). The data are presented in Table XXIII and Figure 14.

This experiment shows that when a shaking apparatus was used, the cell count in the bactone medium was about the same whether or not KOH was used as a moistener. The "KOH cultures" did not exhibit the long lag phase which was shown in previous experiments. The significance of the difference in growth between the "KOH cultures" for the two different media, B' and Sy', will be discussed later.

Chemically defined medium supplemented with certain dicarboxylic acids. These experiments were carried out in order to see whether or not any of the dicarboxylic acids, which are intermediates in the "Krebs cycle," are capable of enhancing the growth rate if the carbon dioxide is removed at the same time. The Dubnoff shaking apparatus was

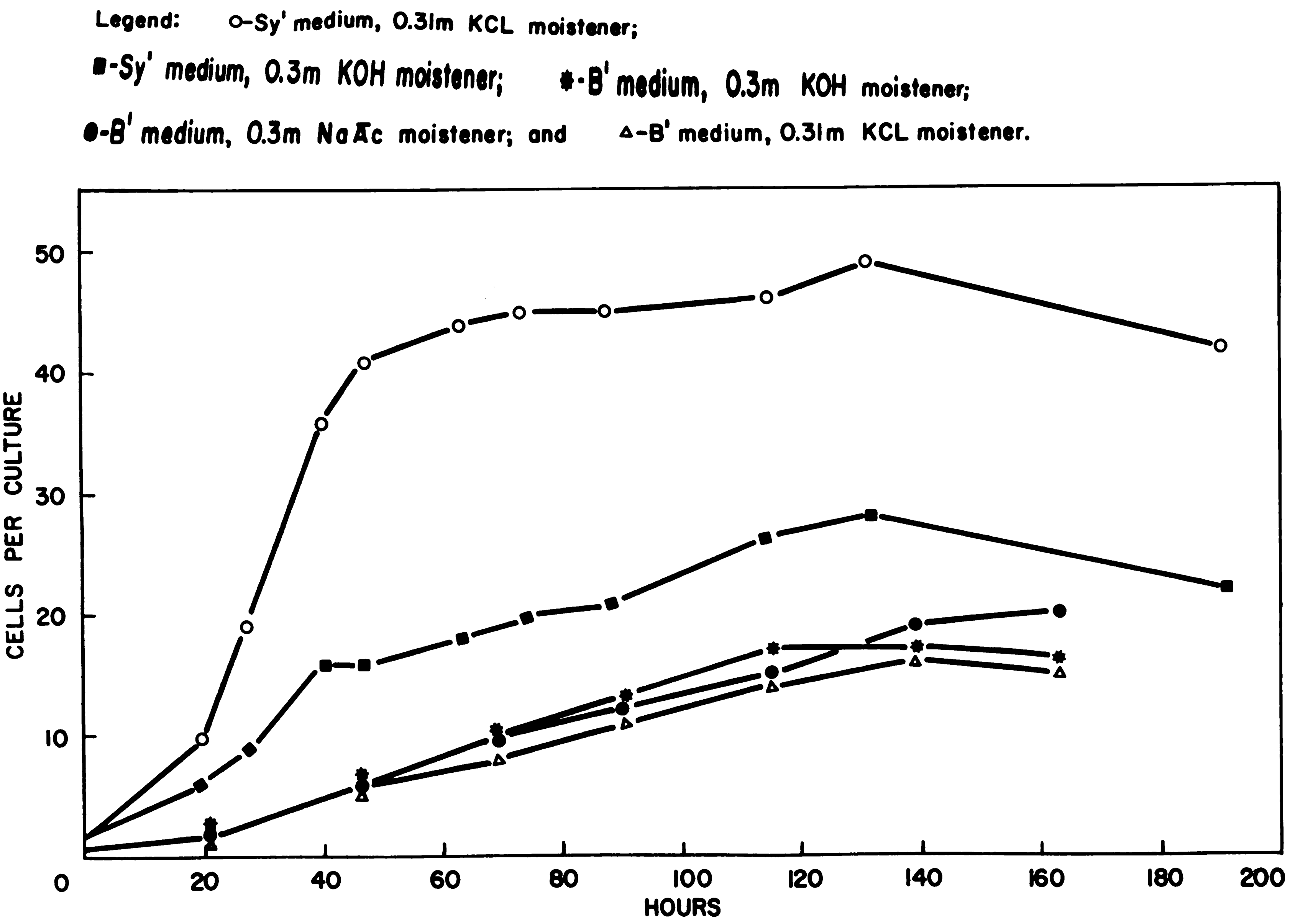
TABLE XXIII

CELL COUNTS USING VAN TIEGHEM CHAMBERS WHICH WERE PLACED IN THE DUBNOFF METABOLIC SHAKING INCUBATOR. MEDIUM USED: B'.

Age of Culture (Hours)	Type of Moistener Used in the Van Tieghem Chamber														
	0.31m KCl					0.3m Na$\overline{\text{Ac}}$					0.3m KOH				
	T1	T2	T3	T4	Av.	T1	T2	T3	T4	Av.	T1	T2	T3	T4	Av.
0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	2	2	2	3	2	2	2	2	1	2	3	2	2	2	2
26	3	4	2	4	3	3	2	2	3	3	3	2	3	3	3
46	6	7	3	6	6	6	4	8	7	6	8	6	7	5	6
69	9	12	4	8	8	8	6	15	12	10	11	9	12	9	10
90	14	14	8	8	11	10	7	16	15	12	15	13	14	12	13
115	16	17	10	12	14	17	12	17	21	15	20	14	16	17	17
139	17	19	11	16	16	18	13	21	26	19	21	14	15	20	17
163	20	13	11	17	15	18	15	22	27	20	15	12	14	24	16
211	17	10	10	16	13	16	16	14	19	16	18	16	15	32	20
259	12	7	3	14	9	11	14	12	17	13	15	8	13	30	16

FIGURE 14

Graphs of the rate of change in cell counts using Van Tieghem chambers and the Dubnoff Metabolic Shaking Incubator. The data were obtained from Tables XXII and XXIII.


Legend: ○-Sy' medium, 0.31m KCL moistener;
■-Sy' medium, 0.3m KOH moistener; ✱-B' medium, 0.3m KOH moistener;
●-B' medium, 0.3m NaAc moistener; and △-B' medium, 0.31m KCL moistener.
CELLS PER CULTURE
0
10
20
30
40
50
20
40
60
80
100
120
140
160
180
200
HOURS

again used and filter paper inserted within the Van Tieghem chamber. The moisteners were 0.31 molal KCl (control) and 0.3 molal KOH (experimental). The following carboxylic acids were added to the chemically defined medium (Sy') so that they were present in 0.02 molar quantities: succinic acid, oxalacetic acid, malic acid, fumaric acid, aspartic acid and alpha-ketoglutaric acid. The pH was varied from 7.4 to 6.0.

The results were almost exactly the same as the growth curves obtained when the dicarboxylic acids were not present. The conclusion is, therefore, that if the lack of carbon dioxide is inhibiting growth, the addition of the dicarboxylic acids mentioned above will not relieve the inhibition.

A further modification of this type of experiment was carried out by adding trans-1,2-cyclopentanedicarboxylic acid (Krishell Laboratories, Portland, Oregon), a succinic acid analog, to the dicarboxylic acid solutions described above so that the cyclopentane dicarboxylic acid has a final concentration of 0.04 molar. Seaman and Houlihan (1950) showed evidence that the cell membrane of <u>Tetrahymena</u> was not permeable to the dicarboxylic acids involved in the "Krebs cycle." According to his observations, the addition of trans-1,2-cyclopentanedicarboxylic acid to a medium containing succinate showed that some of the succinate was removed from the culture medium.

In the experiments conducted using Van Tieghem chambers this succinic acid analog had no effect on the growth rate of the "KOH cultures." In other words, the presence of trans-1,2-cyclopentanedicarboxylic acid did not relieve the inhibition of growth; the inhibition of growth may or may not be due to a lack of carbon dioxide.

6. The use of a modified Van Tieghem chamber to remove carbon dioxide from the environment of the cell.

The apparatus used in these experiments is pictured in Plates 2 and 3 and was described in detail in "Materials and Methods," Part D,4. The culture media are the same as used in the previous experiments. The results of these experiments are summarized in Table XXIV and Figure 15.

Table XXIV (Sy' medium) shows that at 90 hours subsequent to inoculation, the number of cells in the control cultures was over four times as great as in the experimental cultures. In fact, even if the experimental culture was inoculated with two organisms while the control culture contained only one organism (Sy' medium, T3), the latter cultures possessed about three times as many cells as the experimental cultures after 90 hours.

If the Sy medium was used, the maximum number of organisms in the experimental cultures was about one sixth as great as in the control cultures. The additional glucose and sodium acetate, which is present in the Sy medium, apparently enhanced the growth of the control cultures, but had little effect on the experimental cultures.

In contrast to the above results, the experimental cultures grew as well as the control cultures if bactone medium (B) was used. Figure 15 shows that the growth curves for the experimental cultures and the control cultures were very different if Sy' medium was used,

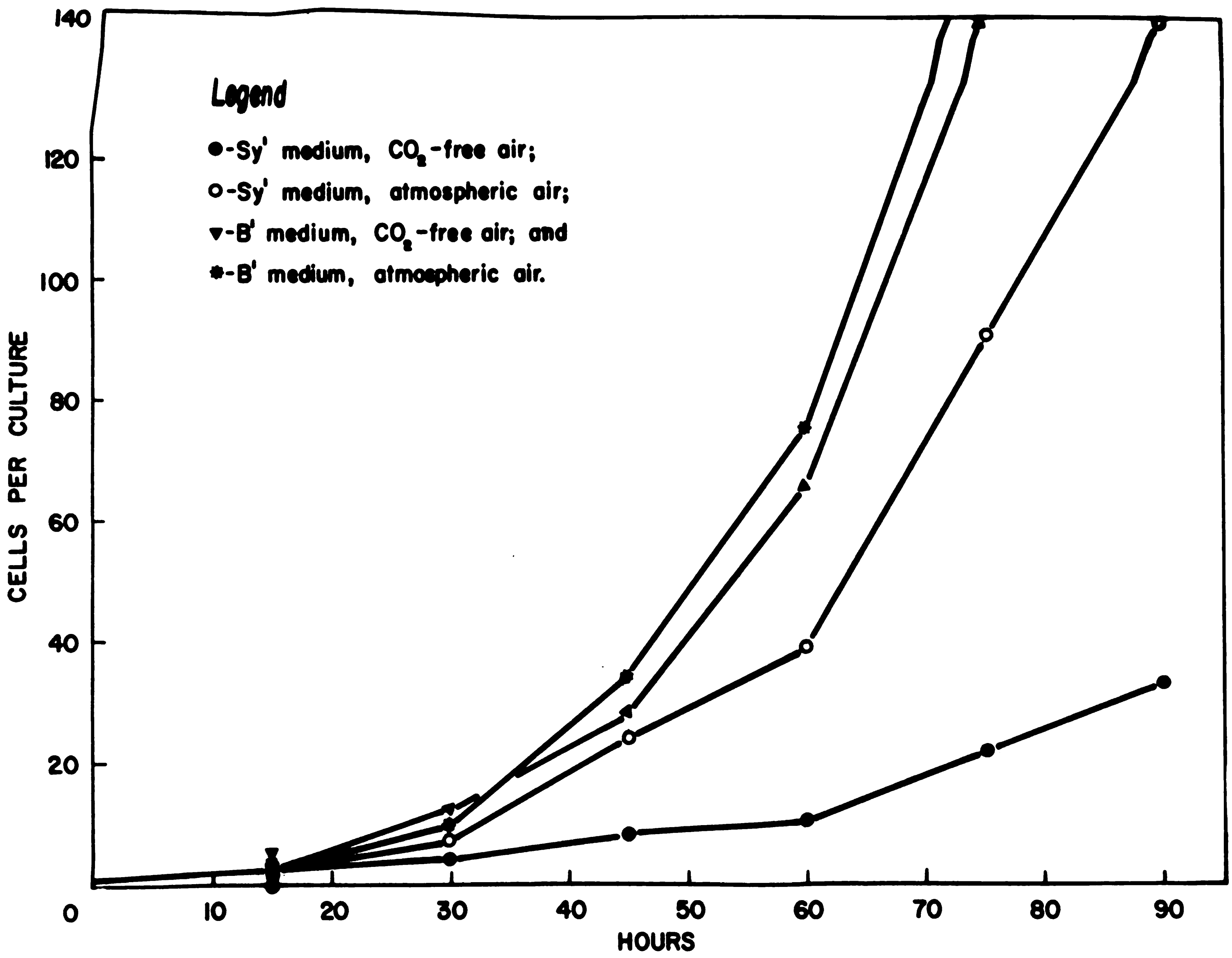
TABLE XXIV

CELL COUNTS USING THE MODIFIED VAN TIEGHEM CHAMBER, AS PICTURED IN PLATES 3 AND 4, WITH VARIOUS TYPES OF CULTURE MEDIA

Medium	Age of Culture (Hours)	Experimental (CO_2-free air)				Control (Atmospheric air)			
		T1	T2	T3	Av.	T1	T2	T3	Av.
Sy'	0	1	1	2	1	1	1	1	1
	15	2	1	3	2	2	4	1	2
	30	3	2	6	4	7	9	4	7
	45	6	2	12	8	23	24	25	24
	60	9	4	17	10	37	33	46	39
	75	19	7	39	21	93	76	102	90
	90	33	21	46	33	152	127	143	140
Sy	0	1	1	1	1	1	1	1	1
	15	5	3	-	3	5	4	-	4
	30	-	-	7	7	-	-	9	9
	45	8	11	13	11	19	16	21	19
	60	-	-	21	21	-	-	52	52
	75	11	26	36	24	112	142	170	141
	90	34	43	58	45	340	276	320	312
B'	0	1	1		1	1	1		1
	15	2	-		2	2	-		2
	30	5	4		5	6	15		9
	45	18	14		16	31	49		32
	60	-	-		-	-	-		-
	75	58	43		50	142	176		122
	90	-	93		93	-	220		220
B	0	1	1		1	1	1		1
	15	4	2		3	3	3		3
	30	12	-		12	10	-		10
	45	34	23		28	29	38		34
	60	63	67		65	57	92		75
	75	-	143		143	-	171		171
	90	-	320		320	-	360		340

FIGURE 15

Graphs of the rate of change in cell counts using the modified Van Tieghem chamber pictured in Plates 3 and 4. Data were obtained from Table XXIV.


Legend
●-Sy' medium, CO₂-free air;
○-Sy' medium, atmospheric air;
▼-B' medium, CO₂-free air; and
✱-B' medium, atmospheric air.
140
120
100
80
60
40
20
0
CELLS PER CULTURE
10
20
30
40
50
60
70
80
90
HOURS

whereas bactone medium (B) produced growth curves that were essentially similar. The significance of the different results obtained when different media (B and Sy') were used, will be discussed later.

Dicarboxylic acids. Experiments utilizing the dicarboxylic acids of the "Krebs cycle" were set up in a manner similar to those in Part 4. Two-hundredths molar concentrations of succinate, oxalocetate, malate, fumarate, aspartate and alpha ketoglutarate were added to the Sy' medium. Additional cultures were set up in which 0.04 molar trans-1,2-cyclopentanedicarboxylic acid was added in addition to the dicarboxylic acid solutions described above.

The results of these experiments revealed that the experimental cultures containing the dicarboxylic acids did not grow any better than the experimental cultures in the previous experiment. In other words the reduced growth rate, using Sy' medium, produced by CO_2-free air could not be relieved by the addition of the dicarboxylic acids listed above, nor did the addition of trans-1,2-cyclopentanedicarboxylic acid produce any further modification of the growth curve.

The results of the last series of experiments, in which the modified Van Tieghem chamber was used, seemed to indicate that the growth rate of Tetrahymena pyriformis W was greatly reduced if the carbon dioxide was removed from the environment of the cell.

DISCUSSION

A. OBSERVATIONS ON GROWTH IN TETRAHYMENA

Hall et al. (1935) reports on error of about 5 per cent when the Sedgewick-Rafter chamber is used to measure population densities of protozoa. Richards (1941, p. 518) states that the chief source of error of this method depends on how closely the sample represents the population. This, of course, is the perennial problem in any sampling procedure. Allen (1921), Berkson et al. (1935), and Serfling (1949) have discussed the statistical errors inherent in sampling techniques as they apply to biological problems.

Experiments in this study in which the Sedgewick-Rafter chamber was utilized were carried out in order to correlate direct cell counts with other methods of growth measurement. No specific attempt was made to evaluate the use of the Sedgewick-Rafter chamber by itself because its limitations have been thoroughly discussed by Whipple (1927). Richards (1941, p. 518) points out that the Sedgewick-Rafter chamber has long been the standard type of growth measuring device for Protozoa.

Monod (1949) discusses the fact, that although there is widespread use of optical techniques, not enough efforts have been made to check them against direct estimation of cell concentrations or dry-weights. The results of this study seemed to indicate that a direct cell count and the optical density of the culture medium are not closely correlated. In fact, for cultures grown in bactone medium, the ratio of the cell count to the optical density measurements varied from a minimum of 2.58 to a maximum of 8.00 during the first 264 hours. On the other hand, the addition of a small amount of thiamine (0.2 gamma per milliliter) to the bactone medium, produced a variation of the same ratio from a minimum of 4.90 to a maximum of 6.00 during the same number of hours. That is, the variation of the culture medium by the addition of just one vitamin has markedly affected the ratio between the cell count and the optical density. The significance of this is that although the correlation of the cell count and the optical density may be fairly good for one type of medium (e.g., thiamine enriched bactone), the correlation may be very poor if the medium is altered by a slight degree. In all of the types of media studied, however, the ratio between the direct cell count and the optical density of the culture medium never approached anything like a proportionality constant. If these two variables, i.e., cell count and optical density, are directly proportional to each other, then the expression "cell count/optical density = K" (where K is a proportionality constant) should hold. Elliott (1949) maintains that the photometer measurements are indicative of cell volume rather than cell number. He also states that the absorption of light is a function of both the size and the contents of cells. The problem, of course, is: Just what does the optical density of the culture indicate? The results of this study would agree that the optical density is probably not a good measure of the cell count. In addition, one must ask: If the optical density is a measure of cell volume and cell content, as maintained by Elliott, is there any way that one could decide which of the variables is being measured?

Experiments in this study showed that the optical density of a bactone medium was not correlated to the total mean protoplasmic area of the culture. The ratio of these two quantities increased from 57.14 at 73 hours to 404.76 at 264 hours. This is about a seven-fold increase. The same ratio in a thiamine enriched bactone medium varied from 57.23 at 73 hours to 90.75 at 264 hours--the increase being by a factor of 1.6. Here again the expression "optical density/total mean protoplasmic area = K" does not hold. It must be admitted that there are inherent errors in the method used to determine the total mean protoplasmic area. It would be difficult to believe, however, that the errors would be of the magnitude to produce a variation in the ratio equivalent to that which was found. Probably an important error in the estimation of protoplasm was the use of cell area instead of cell volume. Popoff (cited by Richards, 1941, p. 521) calculated cell volumes of P. caudatum as equal to $4\pi LBT/24$, where L, B and T are equal to length, breadth and thickness, respectively. This formula assumes the cell to be a spheroid. Since it was difficult to see how accurate measurements could be made on the cell thickness, the assumptions made by Popoff were not followed.

Since neither the protoplasmic area nor the cell count seem to be correlated to the optical density, one should consider the "cell content" as a possible source of optical density. Stier et al. (1939) reports that in yeasts a change from 0.170 to 0.417 occurred in the optical density within six hours after the addition of 5 per cent glucose to the suspension. He maintained that there was no significant change in the total cell count during the six hours but that an increase in the opacity of the cells was evident. The above reference suggests that cell content may be a very important variable that should be considered in optical density measurements. In our study, the only obvious variation in the opacity of the protoplasm occurred when sodium acetate was used as a moistener in the Van Tieghem chambers. In contrasts to the experiments of Stier et al., given above, are the results of Gause (1934). In separate cultures of P. aurelia and P. candatum, with the same culture medium, he reported that the same total volume of protoplasm was produced by each species although a direct cell count revealed that there were over twice as many P. aurelia as P. candatum. It may be argued that a change in cell content constitutes a change in growth and consequently if the optical density can depict a change in cell content then this should be acceptable as a measurement of growth. The fact must be considered, however, that a change in the opacity of the cell may be due to only a small quantity of a substance which is very opaque whereas a different substance, even though present in a greater amount, could conceivably transmit a high percentage of the incident light. Thus one should carefully qualify any statement which attempts to correlate an increase in cell opacity with an increase in cell growth.

Stier et al. (1939) in his studies on the opacity of yeast cells made the observation that there was no change in the optical density of the suspending medium during the duration of the experiment. In our studies it was realized that in addition to the variables already stated, namely, cell count, cell volume and cell content, one should always consider the possibility that the optical density of the culture supernatant might change significantly during the course of the experiment. This seems to be an easily overlooked possibility although Elliott (1949) mentions that a gradual change in the color of the medium often occurs during incubation. He mentions as possible causes that this may be due to the consumption of certain foods, accumulation of wastes, or a shift in pH. Our measurements of the optical density of the supernatant strongly indicate that there are significant changes in the optical density of the supernatant as the culture ages. A study of Figure 11 reveals that this optical density change varies with the type of medium used. In addition to the fact that the optical density of the supernatant appears to be a variable, and thus should be considered in any optical measurements of the cultures, there are at least three additional observations that should be pointed out in connection with this fact.

(1) In several cases (Tables XI, XII, XIV) it appears as though optical changes occurred in the supernatant prior to any significant increase in the number of cells in the culture. When a 2 per cent bactone medium was used, for example, the optical density of the culture medium was actually due to optical changes in the supernatant. This is evident from the fact that the optical density of the supernatant is equal to the optical density of the culture medium. It is quite obvious from this observation that one could erroneously interpret optical density changes as an increase in protoplasm or cell content if one ignores the possibility of optical changes in the supernatant. This would be particularly important if one were comparing the stationary or lag phases produced by different types of media because, as described

above, important changes occur in the supernatant during these phases of the growth curve.

(2) As was pointed out from Figure 11, the optical density of the supernatant was related to the presence of cells in the culture medium. That is, fresh culture medium did not increase in optical density, but usually showed a slight decrease (Tables XI through XIV). However, the optical density was not directly proportional to the increase in the number of cells because only a slight increase was shown in the optical density of the supernatant during the logarithmic phase of growth. One would suspect, therefore, that the initiation of optical changes in the supernatant was dependent on the presence of cells, but that the subsequent changes in the supernatant seemed to occur at a rate independent of the increase in cell count. In many cases (Figures 7 through 10), the graph of the optical density of the supernatant was nearly a straight line for the first 200 hours. This occurred at a time when the graph of the cellular growth resembled the usual sigmoid curve. It is difficult to account for this pecular situation. One might question the accumulation of optically sensitive materials in the supernatant. For example, it could be hypothesized that the centrifugation of the cells would produce some cell breakage which would release materials into the supernatant. Seaman (1955, p. 136) reported evidence that a small amount of cell rupture occurred during centrifugation procedures and thus recommended centrifugal speeds of only 750-1000 x g. It is estimated that a force of about 1000 x g was used in our experiments. Even if one admits that centrifugation can become a factor in this type of experiment, there are difficulties

which cannot be explained. For example, it is difficult to see how centrifugation could be responsible for the increase in optical density produced in the supernatant during the first 48 hours (Table XI) when the number of cells per milliliter is very small. Secondly, it would seem that centrifugation should produce a proportionately greater amount of optical density change in the supernatant when the maximum cell count was reached. That this is not the case can be seen if the graphs of the optical densities of supernatant and culture medium are compared (Figures 7 through 10).

(3) Bactone medium "E" produced a maximum growth which was over three times as great as a non-enriched bactone medium. It was surprising, however, to see that the optical density for the supernatant of the bactone "E" medium was usually less than the supernatant of the bactone medium (Figure 11). In fact, at 160 hours subsequent to inoculation the optical density of the bactone medium "E" supernatant was only one-half as great as was found in the non-enriched bactone medium. One would assume that if the optical density of the supernatant is due to some substance excreted by the cells, then an increase in cell population should produce a corresponding increase in the excreted substance. The converse of this seems to be the case, however, because bactone medium "E" which produced the greatest cell growth also produced the supernatant with the smallest optical density. On the other hand, one might speculate that the additional growth factors available in bactone medium "E" altered the metabolism of the cells in a way that would ultimately change the nature of the excretory materials. Seaman (1949) reported that lipid material is excreted into the culture

fluid. He states that as the population increases there is a gradual increase in the quantity of lipid materials in the culture medium. Once the stationary phase is reached, however, there is a rapid decline in the non-cellular fatty acids, but after a short time they again accumulate in the culture medium. Fennell (1951) demonstrated similar changes (exclusive of the last mentioned increase subsequent to the stationary phase) by histochemical techniques. One might suspect that the secretion of the fatty acids might be related to an antibacterial property of unsaturated fatty acids, but McKee _et al._ (1947) finds no bacteriostatic effect of the supernatant and thus assumes that the secreted fatty acids are saturated. Seaman (1949) believes that the cells may lose their ability to synthesize fatty acids soon after maximum growth is reached and that the sharp decline of the lipid material present in the culture medium during pre-maximum growth may be due to the reabsorption of the fat by the older cells. The increase in the optical density of the supernatant may possibly be due to the increase of excreted fatty acids. Our optical density measurements, however, did not show any sudden decrease at the point where Seaman claims that the supernatant fatty acid rapidly declines. Furthermore, the accumulation in supernatant fatty acid appears to be much too rapid to be compared to the much slower change which occurs in the optical density of the supernatant. It seems peculiar that free-living protozoa would possess this capacity of excreting a possible food source, which would, under normal conditions, be rapidly diluted and lost to the organism. Wu and Hogg (1952) show that small, but possibly significant, amounts of alanine, aspartic acid, glutamic acid, glycine and tyrosine are also excreted by Tetrahymena. Apparently the primary excretory product of protein metabolism is ammonia although Nardone and Wilber (1950) show that urea is excreted during the early phases of growth. It is interesting to note that ammonia accumulation appears to be somewhat directly proportional to time and thus the graph of ammonia accumulation is a straight line. Consequently, ammonia accumulation and the optical density change in the supernatant at least have this much in common. The possibility that an extracellular enzyme may be acting on a substrate in the culture solution should be considered, but an extracellular gelatinase (Lwoff, 1932) capable of liquifying gelatin, is the only extracellular enzyme reported for Tetrahymena.

It would seem that the only way to control the effect of the optical density of the supernatant upon growth estimation, is to use the culture supernatant, instead of fresh medium, as the standardization solution. If this is done, the experiments are better controlled since one more variable is removed from consideration. It was found (Table XVI) that the ratio between cell count and optical density was quite constant during the stationary and later phases of growth if the culture supernatant was used as the standardization solution. This would indicate that optical density, if measured in this way, is a good estimation of the total cells. It seems to be an indication also that the cell dimensions are fairly constant during the stationary phase of growth. This deduction would be in good agreement with the findings of Ormsbee (1942) who showed that the cell count was quite constant during the stationary phase and that there was neither cell division nor cell death. It was found from our studies that the correlation between cell count and optical density was unsatisfactory during the early and late phases of the population curve. A possible explanation for this lack of correlation can be found in an analysis of the change in cell dimensions during growth. Ormsbee states that the cells are about 15 per cent shorter during the logrithmic phase than during the stationary phase and that there is a slight increase in length during the stationary phase. The observation is well known that the cells become much smaller during the final stage (phase of decline) of the population curve. This decrease in cell length during both the logrithmic phase and the phase of decline would partially account for the lack of cell count-optical density correlation found during these phases.

As pointed out by Monod (1949), dry-weight measurements provide a very good estimation of total growth, but are time consuming and provide very little information concerning the early phases of growth. For these reasons, dry-weight determinations are not commonly used for estimations of the rate of growth in protozoa although certain measurements (e.g., Q_{O2}) are often expressed in terms of dry-weight. Ormsby (1942) showed that the dry-weight (milligrams per million cells) varied from 5.85 to 8.34 during the stationary phase. Our results showed a variation between 6.2 and 7.4 (milligrams dry-weight per million cells) during the stationary phase. These values are of the same order of magnitude as Ormsby's.

Van Tieghem chambers provide a very convenient means for culturing organisms on which accurate cell counts can be made. Growth curves obtained during the experiments on carbon dioxide fixation were quite accurate in that the growth of clone cultures in the Van Tieghem chambers could be easily followed during the important lag phase. It is the author's opinion that Van Tieghem chambers provide an excellent growth measuring device for several reasons. First, the direct cell count is extremely accurate in that no samples are taken as representing the entire population nor are errors accumulated by making dilutions. The early phases of growth can be easily followed and exact studies can be made on the time factors related to cell divison. Monod (1949) points out that not enough information is ever available for the initial stationary phase and the lag phase because of the usual difficulty in making measurements at this time. The Van Tieghem chamber is particularly well suited, then, for the task of analyzing important variations during the early phases of growth. This method might be put to good advantage, therefore, in studies which deal with the formation of adaptive anzymes. At maximum growth, during the stationary phase, the cells are usually too numerous to be counted individually. Several methods can be used to obtain good direct cell counts during maximum growth: (a) count specific areas of the culture using a Whipple micrometer; (b) use a 0.35 per cent agar nutrient solution to inhibit the fast movement of the cells (Plate 7); or (c) obtain photomicrographs with Kodak tri-X film at 1/1000th of a second (Plates 5 and 6). It is admitted that the Van Tieghem chamber provides a specific method for measuring cell numbers. Consequently, results can, and should, be reported as "cell numbers" and not by the more general term of "growth." As pointed out by Sullivan (cited by Taylor, 1941): "It is much easier to make measurements than to know what you are measuring." When one knows that he is measuring "cell counts" *per se*, that is enough of a measurement in intself. Secondly, the Van Tieghem chambers permit (and perhaps, more important, demand) a direct examination of the organisms at all times. Any morphological changes in the cells during growth or cell divison are likely to be noticed. It is a common criticism that many of today's experimental procedures are too indirect and, therefore, methods should be found to stay as close to the living organism as possible. The author made many interesting observations on the behavior of *Tetrahymena* during the course of these studies because of the necessity of making direct counts on live cells. Thirdly, any changes that occur in the cell dimensions or in the opacity of the cells can be quickly noted if the Van Tieghem chamber is used as a culture chamber. Accurate estimations of the cell dimensions can be made with the Whipple micrometer disc. It was observed during the studies on carbon dioxide fixation that the opacity of the cells is indeed a variable in some cases. The continuum from extreme translucence to extreme opacity of cells was observed in the course of the experiments. It is doubtful that any particular correlations concerning opacity would have been made if the cultures had not been grown in Van Tieghem chambers. Fourthly, the fact that optical density measurements do not differentiate between dead and living cells or cell debris can be avoided by this method. As the culture ages, the dead cells sink to the bottom center of the drop and accumulate there. The cell counts taken, therefore, can be selective for only live cells. This is an advantage over the Sedgewock-Rafter method, which usually utilizes specific dilutions of killed cells for the direct cell counts.

The primary disadvantages to the Van Tieghem chambers are: (1) the time required to set up the cultures, and (2) the small number of cells used for inoculation. The first criticism can only be weighed against the advantages offered by this type of culture method. The choice of culture method should ultimately depend only upon which method is best able to evaluate the variables under consideration. The second criticism is a valid one in that it is often the desire to use clone (single cell) cultures in the Van Tieghem cultures, a practice which can show individual variations among organisms. This variation can be avoided quite well by selecting cells from the logrithmic phase of the stock culture. It was the author's observation that consistent growth curves were obtained if only actively swimming cells were chosen for the clone cultures. That is, if the procedure described in the "Materials and Methods" is used, one is able to observe and select the cells before they are sealed in the Van Tieghem chamber. In this way any cells that appear to be injured in any way can be discarded. The clone culture has the advantage, of course, in that it provides a high degree of genetic control in the resultant population. Similarly, if any morphological or biochemical mutants are, by chance, selected as the initial inoculum, the entire resulting population will be a pure culture of the new strain.

In summary, it is the author's opinion that the Van Tieghem chamber can be used to a very good advantage as a growth measuring device for the reasons stated above. In cases where Van Tieghem chambers are not desirable, optical density measurements of the culture medium are a good estimation of growth provided the optical density change in the supernatant is taken into consideration.

B. STUDIES ON CARBON DIOXIDE REQUIREMENT IN TETRAHYMENA

The experiments by Jahn (1933) and by Pace and Ireland (1945) agree that Tetrahymena grows very well if CO_2-free air is bubbled through a bactone culture solution. The conclusion drawn from this experiment was that Tetrahymena does not require carbon dioxide for growth. Van Niel et al. (1942) as well as Lynch and Calvin (1952) agree that a considerable amount of carbon dioxide fixation occurs in Tetrahymena. The situation presented in the above paragraph does not portray an inconsistency. In the first place, the ability to fix carbon dioxide does not imply that the fixation of carbon dioxide is an indispensable reaction in metabolism. For example, the fact that an organism has the ability to ferment glucose does not necessarily mean that the organism cannot survive unless glucose is available. On the other hand, it may be possible that: (a) more sensitive methods for carbon dioxide removal might reveal a carbon dioxide requirement for Tetrahymena, and (b) the use of a chemically defined medium, instead of the bactone medium used by Jahn (1933) and Pace and Ireland (1945), might provide different results.

The various methods used in our studies to remove the carbon dioxide produced varying degrees of success. The results from the gas-washing apparatus, for example, were highly variable and inconclusive. We believe the poor results were due to excessive foaming of the medium which was caused by the small bubbles produced by the fritted dispersion cylinders. The use of the fritted cylinders in turn demands the use of a fairly high air pressure to force the air through the cylinders which results in excessive foaming. Perhaps some anti-foaming agent would correct this problem. In an attempt to keep the air pressure as low as possible, that pressure was used which was just in excess of the threshold pressure demanded by the fritted cylinder. Consequently, a slight change in the vacuum used to force the air through the system could easily discontinue the flow.

Experiments which employed various types of desiccators and Petri dishes (Table XVIII) containing KOH, were unsuccessful for a different reason. It is doubtful that one can rely on an efficient removal of carbon dioxide from the culture medium by diffusion alone. Of course the reason that gas-washing bottles are usually employed is that they provide a continual agitation within the medium itself. However, it must be admitted that perhaps the carbon dioxide was completely removed in this type of experiment and that growth was not inhibited as a result. These data would then support the hypothesis that Tetrahymena does not possess a carbon dioxide requirement for growth.

Somewhat different results were obtained (Figures 13 and 14) when the Van Tieghem chambers were used. It was fortunate that one could combine into one experiment an excellent culture method (see Discussion, Part A) with a means for removing the carbon dioxide from the environment of the cells. It is admitted that the results from the various experiments in which KOH was used as a moistener (and carbon dioxide absorber) in the Van Tieghem chambers were not consistent. In the first place, it is evident (Figure 12) that a 0.3 molal KOH solution (or its osmotic equivalent) can greatly inhibit growth by the thermal distillation of water from the culture medium. However, if one uses KOH to absorb carbon dioxide it is assumed that concentrations greater than one per cent KOH should be used. According to Umbreit et al. (1945), under most circumstances a one per cent KOH solution is adequate for Warburg studies, but most workers use between 5 and 20 per cent instead. A 0.3 molal KOH solution is between one and two per cent KOH. That is, in order to obtain a concentration of KOH which will absorb the carbon dioxide, one must use a concentration which is not osmotically equivalent to the culture medium. Consequently, any inhibition of growth due to a deficiency of carbon dioxide, is an inhibition over and above that due to thermal distillation. In other words we are testing for the further inhibition of a growth situation already inhibited by thermal distillation. An additional variable results from the chemical reaction between KOH and carbon dioxide which would change the molality of the KOH and this in turn would possibly change the amount of thermal distillation which occurs.

If isosmotic solutions (0.3 molal) of KOH and KCl are used in the Van Tieghem chambers, the maximum growth for both cultures will ultimately be quite comparable but the increase in the population within the "KOH culture" is much slower (Figure 13). If the experiment has been properly controlled, it is possible to interpret this slow growth as being due to a withdrawal of the carbon dioxide.

An inconsistency occurs, however, when similar experiments are performed using the Dubnoff Metabolic Shaking Incubator (Figure 14). In this situation, there is practically no difference in growth between the experimental (KOH) and control (KCl or Na acetate) cultures if bactone medium is used. However, if Elliott's chemically defined medium (minus glucose and sodium acetate) is used, there is a very significant difference between the experimental (KOH) and control (KCl) cultures. This can be interpreted by postulating that either the bactone medium contains some factor that can substitute for carbon dioxide or that it contains a continual source of carbon dioxide which cannot be completely absorbed by the KOH. Evidence for the latter interpretation can be obtained by testing the bactone solution with barium hydroxide. Upon doing so, even immediately after boiling the bactone solution, a white, cloudy precipitate results. This observation is usually considered indicative of the presence of carbon dioxide. The chemically defined medium, when tested with barium hydroxide, shows a much smaller amount of precipitation. This would seem to indicate, therefore, that the bactone medium has a much greater potential supply of carbon dioxide than does the chemically defined medium. If this is the case, it is possible that the carbon dioxide was not completely absorbed by KOH when bactone medium was used (Figure 14) and thus no essential difference was noted between the growth of the experimental and control cultures. The experiments of Gladstone <u>et al.</u> (1935) support this conclusion. They showed that certain bacteria would not grow in a current of CO_2-free air provided a synthetic medium is used. However, if bactone medium was used, the growth of the bacteria could not be inhibited by CO_2-free air. Winslow <u>et al.</u> (1932) demonstrated a similar situation for <u>E. coli</u> by showing that inhibition of growth by CO_2-free air could not be maintained if the synthetic medium (lactose, ammonium tartrate and ammonium phosphate) was replaced by peptone or a broth containing lactose. Gladstone et al. (1935) also ran thorough tests to show that the removal of carbon dioxide did not alter the pH of the medium.

The papers referred to above support the notion that a bactone medium may be a ready source of carbon dioxide. This would account for the growth of Tetrahymena in bactone medium under conditions designed to remove carbon dioxide (Figure 14). Perhaps this would also account for the experiments of Jahn (1933) and Pace and Ireland (1945) in which they found no inhibition of growth in Tetrahymena when they used bactone medium and CO_2-free air. It must be admitted, however, that experiments performed by the author, in which gas-washing bottles were used to remove the carbon dioxide from air bubbled through chemically defined medium, did not inhibit growth of Tetrahymena. The experimental difficulties with this procedure were discussed earlier.

Results obtained when chemically defined media are used in conjunction with Van Tieghem chambers containing KOH to remove the carbon dioxide, support the hypothesis that carbon dioxide is a growth requirement for Tetrahymena. Even more clear-cut results (Figure 15) are obtained, however, when the apparatus pictured in Plates 2 and 3 is used to remove the carbon dioxide from the environment of the cells. This method enables one to correct the possible variable of thermal distillation which occurs when KOH is used as a moistener in the Van Tieghem chambers. The fact that the air is bubbled through distilled water before entering either the experimental or control chambers seems to provide an excellent degree of control in this type of experiment. Many tests were made on pH, drop-size, and several other possible variables without discovering any apparent sources of error. The fact that good growth occurs in the experimental CO_2-free air chambers containing bactone medium at least indicates that growth can occur in these chambers. In other words, there are not factors (e.g., KOH droplets) present which would inhibit the growth whether carbon dioxide is removed or not. Experiments in which the modified Van Tieghem chamber was used support the hypothesis that carbon dioxide is required for growth of Tetrahymena using a chemically defined medium (Figure 15).

Additional support for any hypothesis can best be obtained by testing predictable consequences of the hypothesis. Heterotrophic carbon dioxide fixation is not well understood, but certain observations have provided clues as to the possible role of carbon dioxide fixation in micro-organisms. For example, it is generally assumed (Kidder and Dewey, 1951) that the incorporation of carbon dioxide in Tetrahymena proceeds by way of the Wood-Werkman reaction:

$$\text{Pyruvic acid} + CO_2 \rightleftharpoons \text{Oxalacetic acid} \qquad (1)$$

It is admitted by most investigators that there may be several pathways by which carbon dioxide might enter into intermediary metabolism. Korkes et al. (1950) have demonstrated an enzyme which catalyzes the reaction:

$$\text{Pyruvic acid} + CO_2 \rightleftharpoons \text{Malic acid} \qquad (2)$$

Ochoa (1951) and Wood (1951) have discussed the relative merits of reactions (1) and (2) but no final conclusion can yet be given. It is indeed possible that carbon dioxide fixation actually occurs in some reaction not yet investigated. However, it has been shown by several workers (Ajl and Werkman, 1948) that bacterial growth, in the absence of any external source of carbon dioxide, is stimulated if the dicarboxylic acids (i.e., succinate, malate, aspartate, alpha ketoglutarate) are supplied in the growth medium. If it is assumed that acetyl-CoA is one of the primary pathways from pyruvate into the "Krebs cycle," it is difficult to imagine why carbon dioxide would be required for the operation of the "Krebs cycle." The "condensing enzyme," which catalyzes reaction (3), has been found in Tetrahymena (Seaman, 1955).

$$\left.\begin{array}{l}\text{Acetyl - CoA}\\ \text{Oxalacetate}\end{array}\right\rangle \xrightarrow{\text{Condensing Enzyme}} \text{Citrate} \qquad (3)$$

Nevertheless, the fact that radioactive carbon dioxide was fixed in the carboxyl group of succinic acid by *Tetrahymena* (Van Niel, 1942) indicates that the "Krebs cycle" might well be linked to carbon dioxide fixation in this organism.

The author's attempts to substitute the dicarboxylic acids in place of carbon dioxide were not successful. This could indicate that the hypothesis that *Tetrahymena* requires carbon dioxide for growth is not valid. In this case, the inhibition of growth produced in the previous experiments would then be due to some variable that was not properly controlled.

On the other hand, one should consider the work of Seaman (1953), in which attempts were made to show the uptake of the dicarboxylic acids by *Tetrahymena*. Seaman cultured the S strain of *Tetrahymena* in synthetic medium at a pH of 5.3 in order to enhance the penetration of succinate in the undissociated form. There is evidence that some succinate is metabolized, but the growth is quite poor due to the low pH. Our studies with the W strain were carried on at pH 6, which was as low a pH as the W strain would tolerate for growth.

Perhaps the succinate will not enter the cell at this pH. It is possible that the use of radioactive dicarboxylic acids will ultimately answer this question. Seaman (1955) states that the W strain apparently possesses a cell membrane impermeable to many compounds which readily penetrate cells of other strains.

Of course it is also possible that the dicarboxylic acids did enter the cell, but that they do not have the capacity to relieve the hypothesized carbon dioxide requirement. Experiments with L. arabinosus (Oginsky and Umbreit, 1954) show that a carbon dioxide requirement can be relieved by additions of three amino acids: aspartic acid, tyrosine and phenylalanine. Bolton et al. (1952) reported the utilization of carbon dioxide in the synthesis of nucleic acids by E. coli. Wood (1946) lists many compounds, other than di-and tricarboxylic acid, in which radioactive carbon, administered as bicarbonate, has been found. In other words, the exact pathway of carbon dioxide fixation has not been established and it is possible that different organisms might possess different pathways. If the "Krebs cycle" operates in Tetrahymena, and it seems probable that it does (Seaman, 1955), a carbon dioxide requirement should involve the intermediates of the "Krebs cycle" as it does in other organisms. It seems evident, therefore, that further studies involving different strains of Tetrahymena as well as new methods to permeate the cell membrane should be carried out in order to clarify the role of carbon dioxide fixation in Tetrahymena. Photosynthetic carbon dioxide fixation is certainly the fundamental chemical reaction of life. Further research may reveal that heterotrophic carbon dioxide fixation is of comparable significance.

Ochoa (1951) summarizes:

> The view is gradually gaining ground that the basic mechanisms of carbon dioxide fixation may be essentially the same in both autotrophic and heterotrophic cells, differing only in the way in which energy is supplied and in the source of hydrogens for reduction. It is conceivable that the photolytic cleavage of water by illuminated chloroplast preparations might under appropriate conditions substitute for the dehydrogenase systems which can be used to supply hydrogens and energy for the reductive carboxylation of pyruvate and alpha-ketoglutarate.

SUMMARY

1. The protozoan, Tetrahymena pyriformis W, was cultured in various types of bactone and chemically defined media.

2. Optical density measurements, direct cell counts and dry weight determinations were made on the growing cultures.

3. It was found that the optical density of the culture medium was not well correlated with either the direct cell count or the total mean protoplasmic area of the culture.

4. Optical density measurements were made on the culture supernatant. There was a definite increase in the optical density of the supernatant during the growth of the culture. The amount of increase in the optical density depended, in part, on the type of culture medium used.

5. Neither the red, green nor blue filter had the capacity to negate the optical density changes that occurred in the supernatant.

6. Of the several types of media used, the supernatant from cultures grown in chemically defined medium appeared to be the most stable and showed the least increase in the optical density of the supernatant.

7. Fairly good correlations between cell count and optical density are obtained during the stationary phase, if the supernatant of the culture medium is used as the standardization for the optical density measurements.

8. No attempt was made to ascertain the nature of the cause for the increase in optical density of the supernatant. The accumulation of ammonia and fatty acids, however, as reported by other investigators, might possibly be linked to the optical changes in the supernatant.

9. Van Tieghem chambers were utilized for establishing clone cultures of *Tetrahymena*. The advantages of using Van Tieghem chambers for growth studies are discussed and detailed information concerning the technique for this type of culture method is given. It is suggested that Van Tieghem chambers be carefully considered as a means for establishing excellent growth curves under certain conditions.

10. The results of Jahn (1933) and Pace and Ireland (1945) were confirmed concerning the presence of growth of *Tetrahymena* in CO_2-free air when their materials and methods were used.

11. Van Tieghem chambers containing KOH to absorb carbon dioxide were utilized to obtain evidence for a carbon dioxide requirement for growth in *Tetrahymena* when the chemically defined medium was used.

12. It was not possible to suppress growth by carbon dioxide removal when a bactone medium was used. This confirms results of other investigators working with bacteria.

13. A Van Tieghem chamber was modified in such a way that the gaseous environment of the culture could be controlled or changed at will. This apparatus was used to remove carbon dioxide from the environment of the cell. Under the conditions of CO_2-free air, growth of *Tetrahymena* was greatly suppressed.

14. None of the "Krebs cycle" acids tested were able to substitute for carbon dioxide as a growth requirement. There was no evidence that the cell membrane was permeable to the dicarboxylic acids even in the presence of trans-1,2-cyclopentanedicarboxylic acid.

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